

Master's Thesis

Analyzing metabolic cell signaling in
adipose tissue through single-cell RNA
sequencing

Eunkyu Bang

The Graduate School
Sungkyunkwan University
Department of Biological Sciences

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Supervised by

Junho Kim

Major Advisor

This certifies that the Master's Thesis
of Eunkyu Bang is approved.

Committee Chair :

Committee Member :

Major Advisor :

The Graduate School

Sungkyunkwan University

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Table of Contents

Abstract

| | |
|---------------------------------------------------------------------------------------|----|
| 1. Introduction | 1 |
| 2. Methods | 6 |
| 2.1 Public dataset acquisition and description..... | 6 |
| 2.2 Preprocessing and formatting for MEBOCOST application | 7 |
| 2.3 Parameter optimization and application of MEBOCOST | 7 |
| 2.4 Statistical analysis of differential gene-expressing cell populations | 8 |
| 2.5 Quantification of metabolite abundances by LC-MS..... | 9 |
| 3. Results | 10 |
| 3.1 Inference of metabolite-mediated cell-to-cell communication via MEBOCOST | 10 |
| 3.2 Data-driven hypothesis on metabolite-mediated communication | 14 |
| 3.3 Identification of cells expressing genes involved in carnitine synthesis | 21 |

| | |
|----------------------------------------------------------------------------------------------|-----------|
| 3.4 Assessment of gamma-butyrobetaine production within individual DHA-responsive cells..... | 25 |
| 3.5 Observation of cells expressing the Bbox1 gene | 29 |
| 3.6 Exploration of carnitine uptake in beige adipocytes..... | 33 |
| 3.7 Profiling of carnitine related metabolite abundances by LC-MS | 37 |
| Discussion..... | 41 |
| References..... | 44 |
| 논문요약..... | 48 |

List of Figures

| | |
|----------------------------------------------------------------------------------------------------------|----|
| Figure 1. Predicted metabolic signaling between adipocyte subtypes | 13 |
| Figure 2. Filtered metabolite-mediated communication network based on DHA-related signaling | 17 |
| Figure 3. Illustration of lysine degradation and hypothesized inter-adipocyte signaling..... | 18 |
| Figure 4. Reproducibility of DHA signaling in independent dataset | 19 |
| Figure 5. Schematic representation of the proposed model..... | 20 |
| Figure 6. Expression of carnitine synthesis genes..... | 23 |
| Figure 7. Conceptual model of gamma-butyrobetaine conversion during carnitine synthesis | 24 |
| Figure 8. Co-expression of DHA uptake and gamma-butyrobetaine synthesis genes | 27 |
| Figure 9. Distribution of Bbox1-expressing cells in white adipocytes | 31 |

| | |
|----------------------------------------------------------------------------------------|----|
| Figure 10. Diagram of rare white adipocytes completing carnitine synthesis..... | 32 |
| Figure 11. Slc22a5 gene expression in beige adipocytes | 35 |
| Figure 12. Carnitine uptake and utilization in beige adipocytes..... | 36 |
| Figure 13. Temperature dependent changes in related metabolites | 39 |
| Figure 14. Summary of the proposed DHA–carnitine–thermogenesis pathway | 40 |

Abstract

Analyzing metabolic cell signaling in adipose tissue through single-cell RNA sequencing

Single-cell RNA sequencing (scRNA-seq) is a powerful tool for studying transcriptomics at the level of individual cells, as it enables the observation of cellular heterogeneity. Advances in scRNA-seq have facilitated cell-to-cell communication research, which is essential for understanding how cells maintain homeostasis through signal transduction. Signal transduction mediates biological processes and is regulated by both proteins and metabolites. While transcriptomic studies have extensively investigated protein-based interactions, metabolite-mediated interactions remain comparatively underexplored. To address this gap, we utilize MEBOCOST, a computational algorithm that infers metabolite-mediated signaling by analyzing the expression of metabolic enzymes and sensor proteins in scRNA-seq datasets. This approach enables the construction of cell-to-cell communication networks based on metabolite-sensor interactions, identifying both the source and target cells involved in signaling.

Our research aims to discover novel signaling metabolites and their associated biological pathways under specific physiological conditions. As a model system, we focused on adipose tissue, a metabolically active organ central to systemic energy homeostasis. We applied MEBOCOST with subsequent gene expression analysis to investigate signaling during cold-induced thermogenesis. Notably, we identified a previously uncharacterized signaling pattern involving dehydroascorbic acid (DHA) in inguinal white adipose tissue. Our analysis suggests that both beige and white adipocytes contribute to carnitine synthesis via DHA-mediated signaling. This integrative strategy enhances our understanding of metabolite-based intercellular communication and provides a framework that can be extended to other metabolically active tissues and disease context.

Keywords : Single-cell RNA sequencing, cell-to-cell communication, metabolite-mediated signal transduction, MEBOCOST algorithm, adipose tissue

1. Introduction

Transcriptomics is the study of the transcriptome—the complete set of RNA transcripts produced by the genome under specific conditions or in a specific cell type. Many transcriptomic studies have utilized bulk RNA sequencing technology, which provides average gene expression in the tissue of interest. In contrast, single-cell RNA sequencing (scRNA-seq) enables the analysis of RNA transcripts from individual cells isolated from tissues or organs (Haque, Engel et al. 2017). It allows for the assessment of transcriptional similarities and differences among diverse cell types. Given these capabilities, scRNA-seq is well-suited for studying cellular heterogeneity, such as distinguishing between cell populations, identifying rare cell types and subtypes, and observing various stages of cell development and cell-specific responses. Furthermore, advancements in high-throughput scRNA-seq technologies, including the 10x Genomics Chromium platform, and the development of diverse computational tools have made the technique more accessible and widely applicable than ever before.

The advancement of scRNA-seq technology has also influenced research on cell-to-cell communication by enabling a deeper understanding of how cells interact (Almet, Cang et al. 2021). When cells receive signaling molecules from neighboring cells via specific receptors, they respond by transmitting intracellular signals, a process known as signal transduction. This process

induces various cellular activities, including cell growth and development, differentiation, maintenance, and immune responses—activities essential to the homeostasis of multicellular organisms. Traditionally, cell-to-cell communication has been analyzed through *in vitro* experiments involving a limited number of cell types and gene sets (Su, Song et al. 2024). Since cell-to-cell communication involves a wide range of cell types and numerous genes associated with diverse signaling pathways, measuring gene expression at single-cell resolution enable us to analyze interactions among diverse cell types more precisely (Jin, Guerrero-Juarez et al. 2021).

Various types of molecules can function as signaling agents in cell-to-cell communication (Alberts 2002). Previously, proteins have been considered the primary signaling molecules, while metabolites were often viewed as mere byproducts of signaling pathways and gene expression. However, emerging evidence like mTOR pathway and TCA cycle suggests that metabolites can play active roles in coordinating biological processes (Saxton and Sabatini 2017, Martinez-Reyes and Chandel 2020). These include not only regulating signaling pathways and gene expression but also influencing protein-related processes such as post-translation modifications and broader metabolic activities (Baker and Rutter 2023). Beyond these roles, metabolites have also been recognized for their involvement in physiological functions, including nutrient sensing and storage, energy regulation, and the synthesis and degradation of molecules in response to environmental conditions (Baker and Rutter 2023). There are several

robust tools (e.g., CellChat) that infer protein ligand–receptor communication offering both interpretability and computational efficiency (Zheng 2022). As the signaling roles of metabolites gain increasing attention, new computational tools are being developed by adapting existing protein ligand–receptor inference frameworks to metabolite–sensor interactions.

One such tool is MEBOCOST, an algorithm designed to predict intercellular communication based on metabolite–sensor interactions (Zheng 2022). It uses scRNA–seq datasets in which cells are annotated. From these datasets, metabolite presence in each cell is inferred by extracting the expression of enzymes involved in metabolic reactions, where metabolites act as either substrates or products. MEBOCOST defines surface receptors, membrane transporters, and nuclear receptor as sensors and extracts their expression levels accordingly. It then, calculates communication scores based on the mean RNA expression levels of enzymes and sensor proteins associated with each metabolite in every cell. Using these scores, MEBOCOST generates cell-to-cell communication networks driven by metabolite–mediated signaling, allowing the identification of active metabolites, their source and target cells, and signaling relationships within complex tissues.

Building on this, our research aim is to identify novel signaling metabolites and their regulated pathways using computational methods. To achieve this, we established the following analytical approach. First, we select publicly available scRNA–seq datasets from dissertations or databases (e.g., National Institutes of

Health (NIH)). Metabolite candidates are identified using the MEBOCOST algorithm, with a particular focus on uncovering previously unrecognized metabolite-based signaling events under specific biological conditions. After determining the candidate metabolites, we investigate biological pathways in which they are involved, using publicly available databases (e.g., KEGG pathway), to understand their biological implications. Once metabolite-mediated pathways are proposed, we perform gene expression analysis to assess whether these pathways are likely to be active in the relevant cell types. This integrative strategy enables a comprehensive investigation of metabolite-based cell-to-cell communication, linking computational inference with biological validation.

To explore this further, we focused on adipose tissue, a key metabolic organ that plays an essential role in maintaining systemic energy homeostasis. Adipose tissue is broadly classified into three main types: white, beige and brown adipocytes. White adipocytes serve as the primary site for energy storage, storing excess nutrients in the form of lipids. In contrast, beige and brown adipocytes play critical roles in temperature regulation through thermogenesis (Allison 2020). Adipose tissue also acts as an endocrine organ, secreting bioactive molecules such as hormones, cytokines, and metabolites that regulate systemic energy balance (Kershaw and Flier 2004). Given its diverse metabolic and signaling roles, adipose tissue serves as an ideal model for investigating metabolite-mediated communication.

In this study, we investigated metabolite-mediated intercellular

communication in inguinal white adipose tissue under cold exposure using a public scRNA-seq dataset. To validate our findings, we applied the same analytical approach to an independent dataset generated under similar conditions. Through this analysis, we identified dehydroascorbic acid (DHA) as a candidate signaling metabolite potentially involved in regulating carnitine biosynthesis in both beige and white adipocytes. The resulting carnitine appears to contribute to thermogenesis in beige adipocytes. Gene expression analysis further supported the functional relevance of this pathway. Overall, our study not only identifies a potential metabolite–metabolite–mediated cell-to-cell communication but also underscores its broader implications in metabolic regulation.

2. Methods

2.1 Public dataset acquisition and description

The original dataset (accession number: GSE184707) was obtained from the NCBI Gene Expression Omnibus. According to the metadata, mice were exposed to three temperature conditions: room temperature, cold exposure at 8 °C for 4 days, and cold exposure at 8 °C for 7 days. Inguinal white adipose tissues were collected, from which pure white and beige adipocytes were isolated. Single-nucleus RNA sequencing (snRNA-seq) was then performed to generate transcriptomics profiles (Wang, Sharma et al. 2024).

The validation dataset (accession number: GSE227441) was also obtained from NCBI. This dataset includes snRNA-seq profiles of mice subjected to thermoneutrality, cold exposure at 6°C for 3 days, and cold exposure at 6°C for 14 days. Although the dataset includes both young and old mice (Holman, Sakers et al. 2024), we did not separate the samples by age in order to focus on the temperature-dependent responses, therefore combined dataset was used in this study. To match the original dataset, which includes only pure white and beige adipocytes, we selected only adipocytes from the validation dataset to ensure consistency in cell populations.

2.2 Preprocessing and formatting for MEBOCOST application

MEBOCOST algorithm was developed in Python and is designed to process datasets generated using Scanpy, a widely used Python-based tool for single-cell RNA sequencing analysis. However, the public datasets we used were processed using Seurat, an R-based analysis framework (Hao, Stuart et al. 2024). To ensure compatibility with MEBOCOST's input format, we converted the Seurat object into the .h5ad file format, which stores annotated data matrices (AnnData object) used in the Python-based workflow (Wolf, Angerer et al. 2018). Specifically, cell metadata—including cell names and associated annotations (e.g., temperature conditions)—and gene expression matrices (e.g., count matrix and gene names) were filtered and exported as csv files using R (v4.4.3), then transferred and converted to .h5ad files using Python (v3.12).

2.3 Parameter optimization and application of MEBOCOST

Converted datasets were divided by temperature conditions to compare metabolic signaling. MEBOCOST (v1.0.4) algorithm provides predefined gene sets related to metabolite-associated enzymes and sensors for both human and mouse. We selected the mouse reference to match the publicly available datasets we analyzed. Since the original and validation datasets used different column names for cell identities, the relevant metadata columns were renamed accordingly. All additional preprocessing steps were followed by the official MEBCOST tutorial (Demo_Communication_Prediction.ipynb in GitHub).

By default, MEBOCOST applies a 0.25% cutoff for metabolite enzyme, sensor gene expression, and presence proportion (i.e., the minimum proportion of cells expressing each gene). To enable broader detection of metabolic signaling, we adjusted the gene expression threshold to 0.1% for both the original and validation datasets, as recommended in the tutorial. The presence proportion cutoff was set at 0.25% for the original dataset and 0.2% for the validation dataset, which was set slightly lower due to the smaller number of cells in the validation dataset. This adjustment was made to better capture potential communication events across a broader range of cells.

2.4 Statistical analysis of differential gene-expressing cell populations

Fisher's exact test was applied to determine whether the proportion of cells capable of producing gamma-butyrobetaine independently-without support from neighboring cells-varied across temperature conditions. Only cells expressing the relevant genes were considered, and Fisher's exact test was performed under the assumption that each cell represents an independent observation—an essential requirement of the test (Kim 2017). The analysis involved two categorical binary variables, temperature condition and gamma-butyrobetaine production.

H0: There is no association between cold exposure and gamma-butyrobetaine production within a single cell.

H1: There is an association between the cold exposure and gamma-butyrobetaine production within a single cell.

P-values were calculated exactly using the cumulative probability, as Fisher's exact test does not rely on approximations and distributional assumptions. All analyses were conducted in R (v4.4.3), and statistical significance is indicated as *** when $p < 0.001$.

2.5 Quantification of metabolite abundances by LC-MS

Male C57BL/6 mice were exposed to one of three environmental conditions for four weeks: thermoneutrality (30°C, n=17), room temperature (22°C, n=16), and chronic cold (6°C, n=20). Subcutaneous adipose tissues (SAT) were harvested, and the abundance of 433 metabolites was measured. Using liquid chromatography-mass spectrometry (LC-MS). Among these, trimethyllysine, gamma-butyrobetaine, and L-carnitine were selected for further analysis. Their relative abundances were statistically analyzed and visualized using GraphPad Prism.

3. Results

3.1 Inference of metabolite-mediated cell-to-cell communication via MEBOCOST

To identify metabolic signaling changes during thermogenesis, we applied MEBOCOST algorithm to a single-nucleus RNA sequencing (snRNA-seq) dataset from inguinal white adipose tissue (Wang, Sharma et al. 2024). The dataset comprises approximately 50,000 pure adipocytes collected from six mice exposed to three different temperature conditions: room temperature (RT), cold exposure for 4 days (CE4), and cold exposure for 7 days (CE7). Under cold conditions, a subset of these cells differentiated into beige adipocytes. For downstream analysis, we focused on the two major adipocyte types (white and beige).

Using MEBOCOST, we inferred communication flows from “sender cells”– based on metabolite-producing enzyme expression–to “receiver cells” expressing the corresponding sensors. We performed this analysis separately for each temperature condition and observed that metabolite-mediated communication was markedly more active under cold challenge (CE4 and CE7) than at RT (Figure 1A–C). To uncover thermoregulation–specific interactions, we focused on signals that newly emerged under cold exposure (CE4 and CE7).

Among them, four metabolite signals cholesterol, iron, adenosine monophosphate (AMP), and dehydroascorbic acid (DHA) were detected under both CE4 and CE7 conditions (Figure 1B and 1C), but not at room temperature (RT; Figure 1A), suggesting that cold stimulation induces distinct metabolite-mediated signaling pathways.

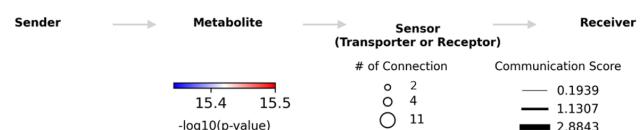
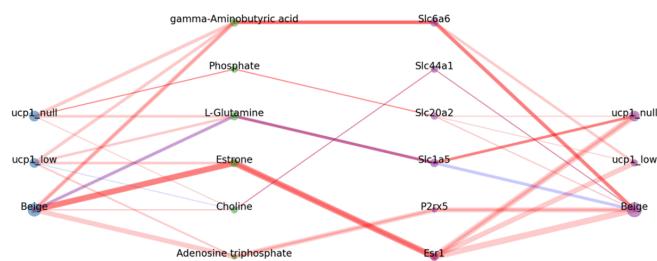
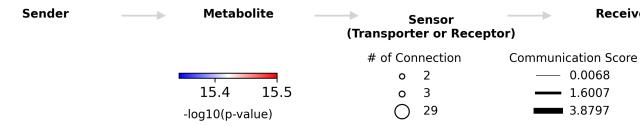
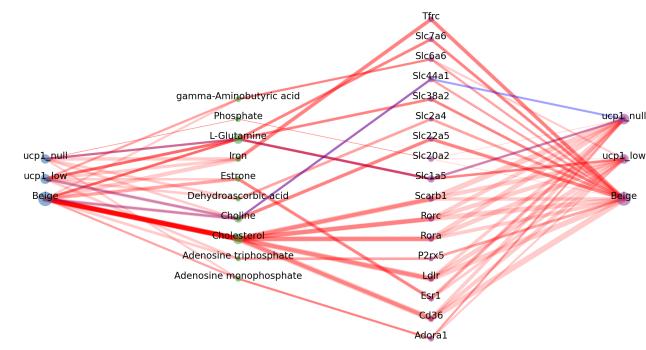
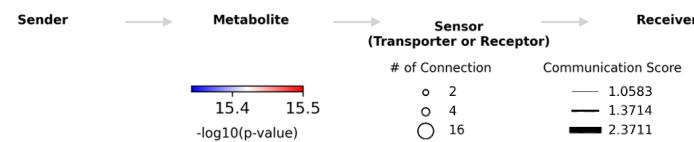
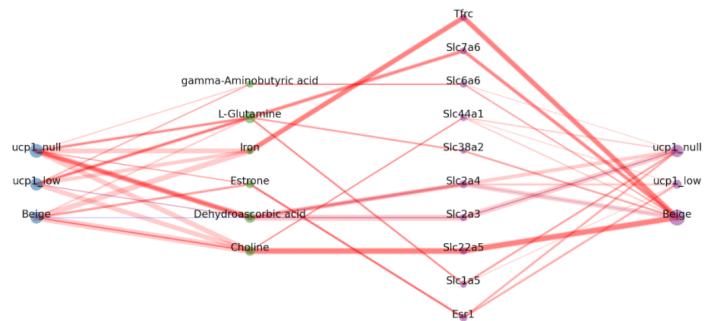
A**B****C**

Figure 1. Predicted metabolic signaling between adipocyte subtypes

MEBOCOST visualizes cell-to-cell communication by displaying sender cells, metabolites, sensor genes, and receiver cells sequentially from left to right. Stronger signals correspond to higher communication scores and thicker connecting lines. All communication pathways shown are statistically significant. The size of each circle represents the number of elements (cells or genes) involved in the communication. **(A)** Room temperature (RT), **(B)** Cold exposure for 4 days (CE4), and **(C)** Cold exposure for 7 days (CE7).

3.2 Data-driven hypothesis on metabolite-mediated communication

We reviewed prior studies for the four candidates' metabolites to guide further analysis. Iron, cholesterol, and AMP have previously been reported to be involved in adipocyte thermogenesis. Iron deficiency has been shown to impair thermogenesis by disrupting iron homeostasis in inguinal white adipose tissue (Yook, Thomas et al. 2021). Cholesterol is involved in thermogenic lipid remodeling and reverse cholesterol transport upon brown and beige fat activation, contributing to a lower risk of cardiovascular diseases (Ying, Tramper et al. 2023). AMP enhances thermogenesis by promoting cAMP signaling and inducing UCP1 expression in brown and beige adipocytes (Rines, Verdeguer et al. 2015). In contrast, no previous studies have demonstrated a role for DHA in this process, indicating a potentially novel role for DHA in cold-induced metabolic signaling.

Based on MEBOCOST communication flow analysis, we selected DHA as a metabolite of interest and observed signaling interactions between white and beige adipocytes (Figure 2). According to MEBOCOST, DHA abundance was inferred from the expression of enzymatic genes, such as Dbh, Gsto1, Gsto2, and Pam, which were detected in both white and beige adipocytes. DHA is predicted to be transported from white and beige adipocytes to other white or beige adipocytes via the glucose transporters GLUT3 and GLUT4, encoded by Slc2a3 and Slc2a4, respectively.

DHA is commonly found in various foods and is known to be derived from vitamin C. Once absorbed into cells, DHA is regenerated into ascorbic acid (AA),

also known as vitamin C. The regenerated AA is essential for the biosynthesis of key biological molecules such as collagen, carnitine, and catecholamine. In addition to its biosynthetic roles, AA enhances the synthesis of peptide hormones and promotes cell survival (Wilson 2002), making it of significant interest in metabolic research.

To investigate the potential signaling roles of our candidate metabolite, DHA, we consulted existing literature and pathway databases (e.g., KEGG PATHWAY Database). Previous studies have shown that brown adipocytes utilize carnitine to support thermogenesis (Ozaki 2011), and that AA regenerated from DHA participates in carnitine biosynthesis via the lysine degradation pathway (Figure 3A), particularly in the liver (Savic, Hodson et al. 2020). Based on these findings, we hypothesized that white and beige adipocytes take up DHA and convert it into AA, enabling them to synthesize carnitine endogenously. Subsequently, beige adipocytes, which share thermogenic functions with brown adipocytes (Ikeda, Maretich et al. 2018), may use the synthesized carnitine during thermogenesis (Figure 3B).

We used a publicly available dataset with similar experimental conditions as a validation set to ensure that our hypothesis was not solely dependent on a single dataset (Holman, Sakers et al. 2024). This dataset includes three groups of mice, those maintained at thermoneutrality (TN), and those exposed to cold for 3 days (CE3) and 14 days (CE14). MEBOCOST algorithm was applied to this dataset to examine whether DHA-mediated communication could also be

detected. Consistent with our original dataset, interactions between white and beige adipocytes via DHA were observed under cold conditions (Figure 4A, B, and C), supporting our hypothesis that both cell types may participate in carnitine synthesis during thermogenesis (Figure 5).

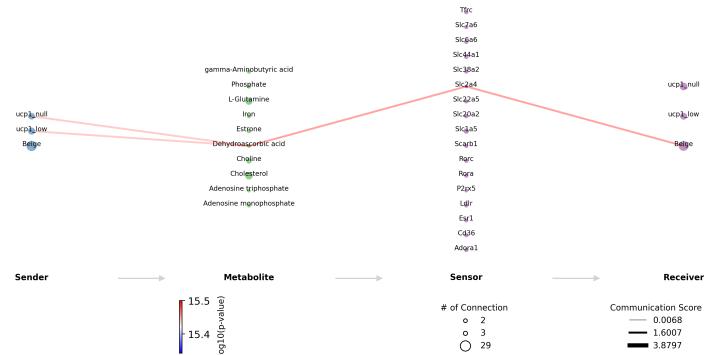
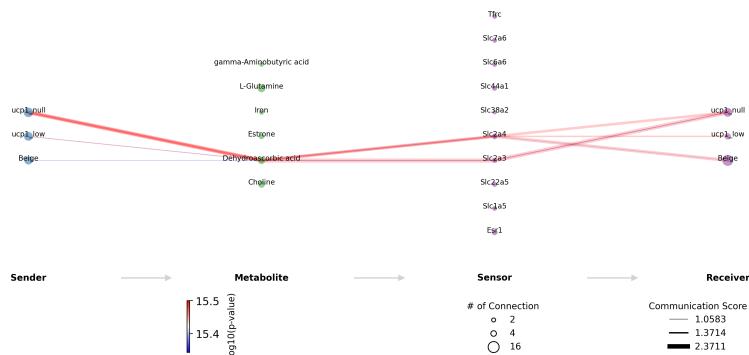
A**B**

Figure 2. Filtered metabolite-mediated communication network based on DHA-related signaling

(A) Acting as signal senders under CE4, white adipocytes transmit DHA to beige adipocytes. (B) Engaging in bidirectional communication under CE7, both white and beige adipocytes act as senders and receivers. The name of clusters, ucp1_low and ucp1_low represent white adipocyte populations.

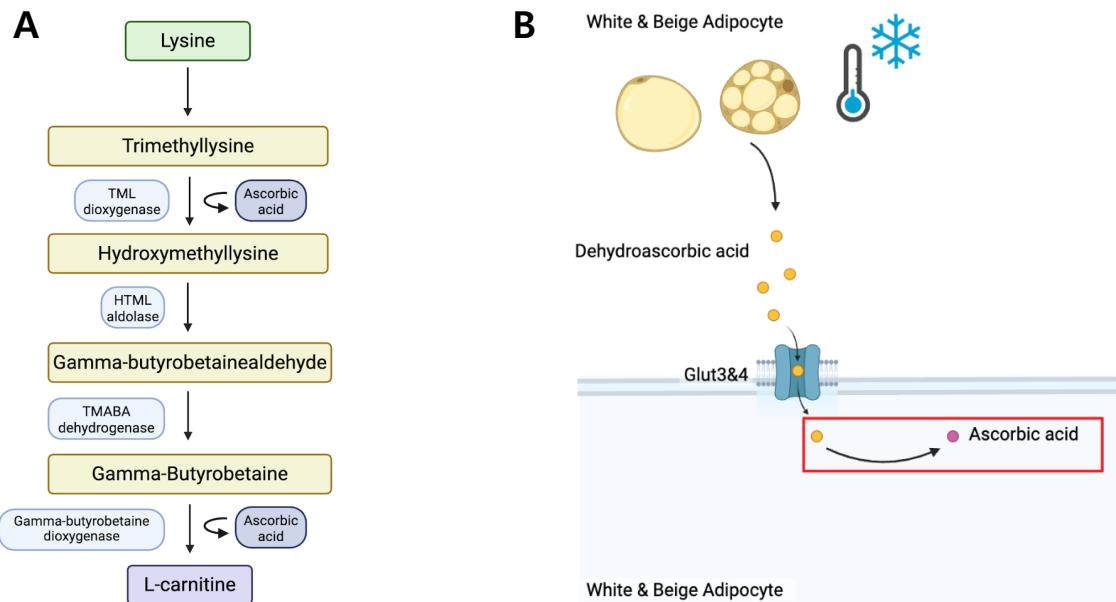
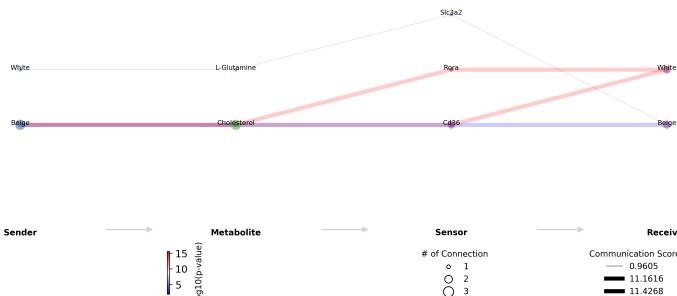


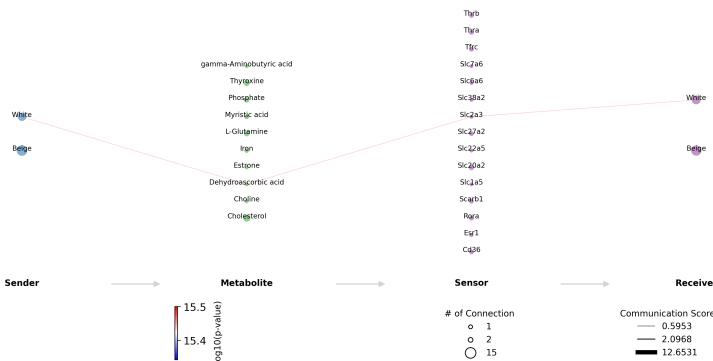
Figure 3. Illustration of lysine degradation and hypothesized inter-adipocyte signaling

(A) Utilization of AA in lysine degradation and carnitine synthesis. Yellow boxes indicate metabolite intermediates, and sky-blue boxes represent enzymes involved in their production. This pathway is primarily active in the liver. (B) Hypothesized DHA-mediated communication between white and beige adipocytes. DHA is proposed to act as a signaling metabolite synthesized and released by both white and beige adipocytes, and sensed by the other white and beige adipocytes through glucose transporters. Both figures were created using BioRender.

A



B



C

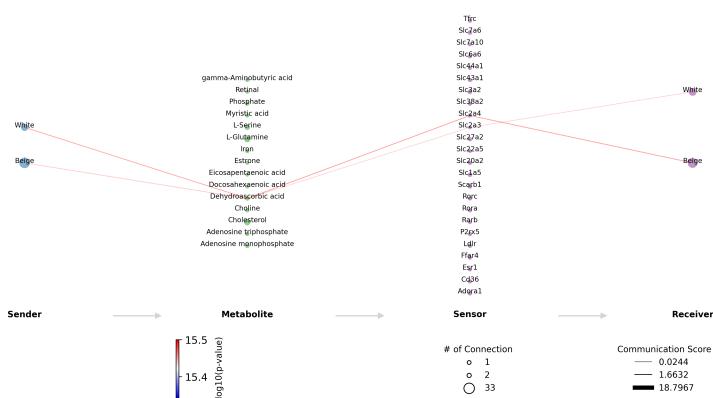


Figure 4. Reproducibility of DHA signaling in independent dataset

MEBOCOST algorithm was applied to a validation dataset under (A) Thermoneutrality, (B) 3 days cold exposure, and (C) 14 days cold exposure. Across all conditions, consistent DHA-mediated communication was observed, with both white and beige adipocytes participating in the signaling process.

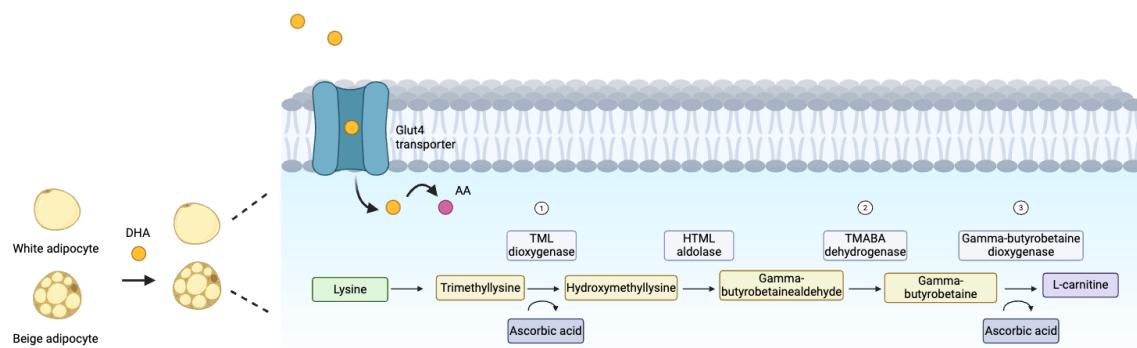


Figure 5. Schematic representation of the proposed model

Conceptual summary illustrating DHA-mediated communication and carnitine synthesis between white and beige adipocytes. Created in BioRender.

3.3 Identification of cells expressing genes involved in carnitine synthesis

The biosynthesis of carnitine from DHA involves several intermediate steps, each catalyzed by specific enzymes. These include TML dioxygenase, TMABA dehydrogenase, gamma-butyrobetaine dioxygenase, which sequentially convert trimethyllysine to carnitine (Savic, Hodson et al. 2020). Specifically, TML dioxygenase catalyzes the hydroxylation of trimethyllysine to hydroxytrimethyllysine; TMABA dehydrogenase converts gamma-butyrobetaine aldehyde to gamma-butyrobetaine; and gamma-butyrobetaine dioxygenase finally converts gamma-butyrobetaine to carnitine (Figure 5).

To verify whether DHA induces this pathway in adipose tissue, we examined the expression of these enzyme-encoding genes in white and beige adipocytes. Both the average gene expression levels and the proportion of cells expressing each gene were analyzed to determine whether they increased under cold conditions. All related genes were referenced based on KEGG pathway for *Mus musculus*. The genes examined included *Tmlhe* (TML dioxygenase); *Aldh* gene family—*Aldh9a1*, *Aldh2*, *Aldh3a2*, and *Aldh7a1* (TMABA dehydrogenase); and *Bbox1* (gamma-butyrobetaine dioxygenase).

In the original dataset, exposure to cold (4 and 7 days) led to increased average expression levels and a higher proportion of cells expressing *Tmlhe* and *Aldh* family genes in white adipocytes (Figure 6A). A similar pattern was observed in beige adipocytes, where *Tmlhe*, *Aldh9a1*, *Aldh7a1* expression also

increased under cold conditions (Figure 6A).

The validation dataset showed consistent trends. In particular, samples exposed to cold for 14 days exhibited increased expression of *Tmlhe* and *Aldh9a1*, *Aldh2*, and *Aldh7a1* genes in both white and beige adipocytes (Figure 6B), supporting the possibility that both cell types can activate the carnitine synthesis pathway up to the production of gamma-butyrobetaine.

However, *Bbox1*, which encodes the final enzyme in carnitine synthesis, showed consistently low expression in both cell types across all datasets (Figure 6A and B), leaving it unclear whether carnitine synthesis is fully completed within inguinal white adipose tissue. We therefore speculate that white and beige adipocytes may generate gamma-butyrobetaine from DHA signal and release it extracellularly for final enzymatic conversion into carnitine (Figure 7).

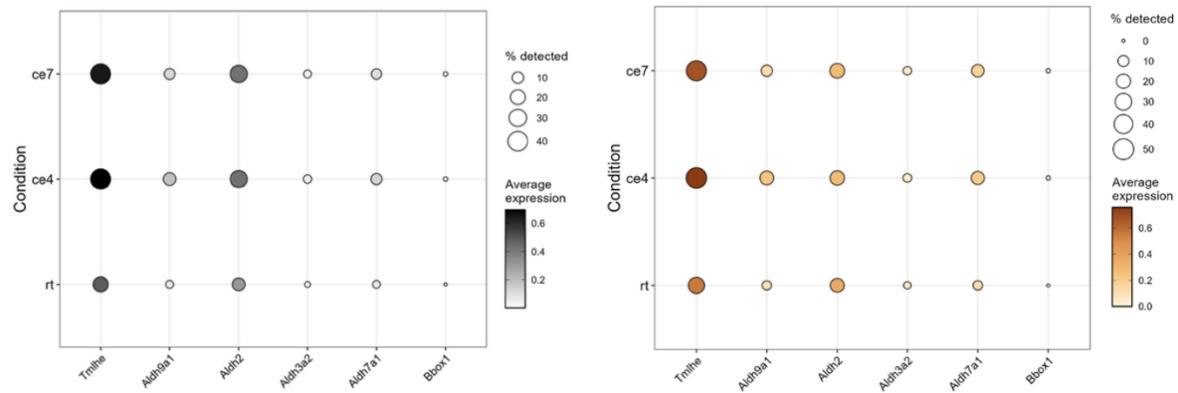
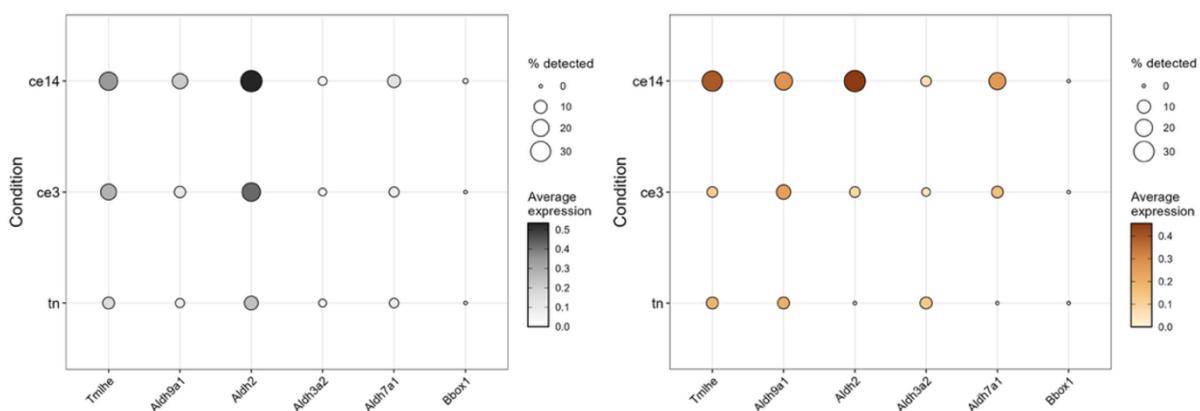
A**B**

Figure 6. Expression of carnitine synthesis genes

Each dot plot displays gene expression patterns based on both the percentage of cells expressing each gene (dot size) and the average expression level (color intensity). **(A)** original dataset and **(B)** validation dataset show aggregated results for white (left) and beige (right) adipocytes, respectively.

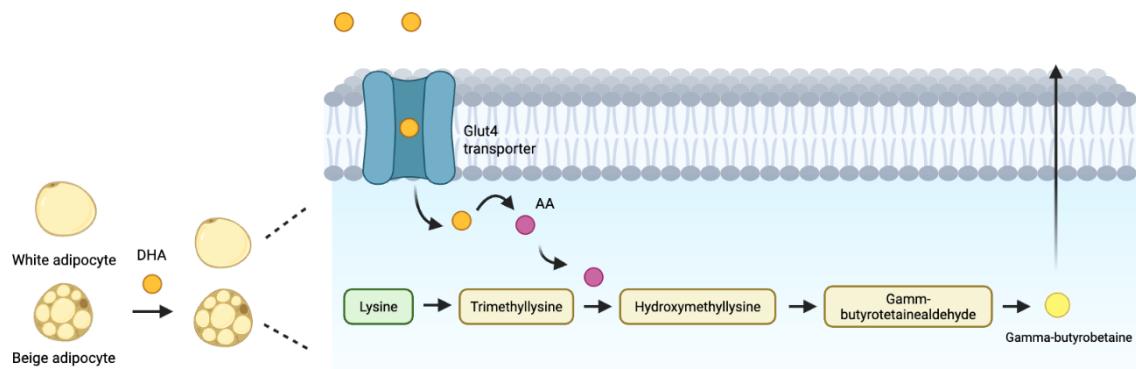


Figure 7. Conceptual model of gamma-butyrobetaine conversion during carnitine synthesis

Due to the absence of a clear increase in Bbox1 expression under cold conditions, this schematic focuses on the upstream steps of carnitine biosynthesis, illustrating the production of gamma-butyrobetaine rather than the full conversion to carnitine. Created in BioRender.

3.4 Assessment of gamma-butyrobetaine production within individual DHA-responsive cells

The synthesis of carnitine from DHA involves multiple enzymatic steps, raising the question of whether a single adipocyte can autonomously perform the entire process. To address this, we conducted an additional analysis to explore whether gamma-butyrobetaine synthesis could occur within individual cells following DHA uptake, rather than requiring coordinated activity across different cell types. We focused on cells co-expressing genes involved in DHA transport: Slc2a3 and Slc2a4 (Rumsey, Kwon et al. 1997, Rumsey, Daruwala et al. 2000) and early carnitine synthesis enzymes: Tmlhe and at least one of Aldh9a1, Aldh2, Aldh3a2, or Aldh7a1, which encode TML dioxygenase and TMABA dehydrogenase, respectively. White adipocytes utilized both Slc2a3 and Slc2a4 for DHA transport, whereas beige adipocytes primarily rely on Slc2a4 (Figure 2). This cell-type-specific expression pattern, when co-expressed with carnitine biosynthetic genes, indicates the potential for intracellular initiation of the pathway under cold conditions.

In the original dataset, both white and beige adipocytes showed a statistically significant increase in the number of cells with this co-expression signature under CE4 and CE7 compared to RT conditions (Figure 8A), supporting the possibility of cell-autonomous activation of the pathway. These findings were further validated in an independent dataset, where white adipocytes exhibited a consistent increase under both CE3 and CE14 conditions, and beige adipocytes

exhibited a similar increase after 14 days of cold exposure, reflecting reproducible across datasets (Figure 8B).

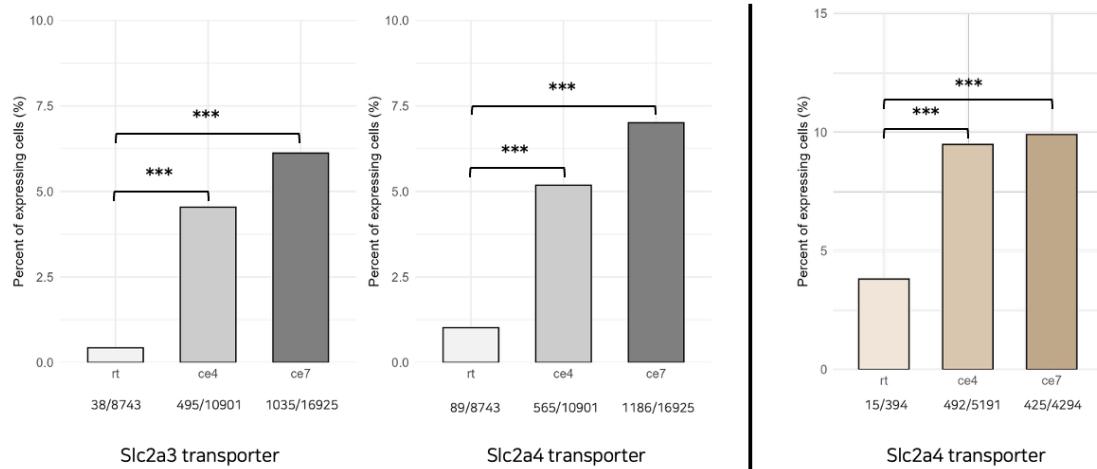
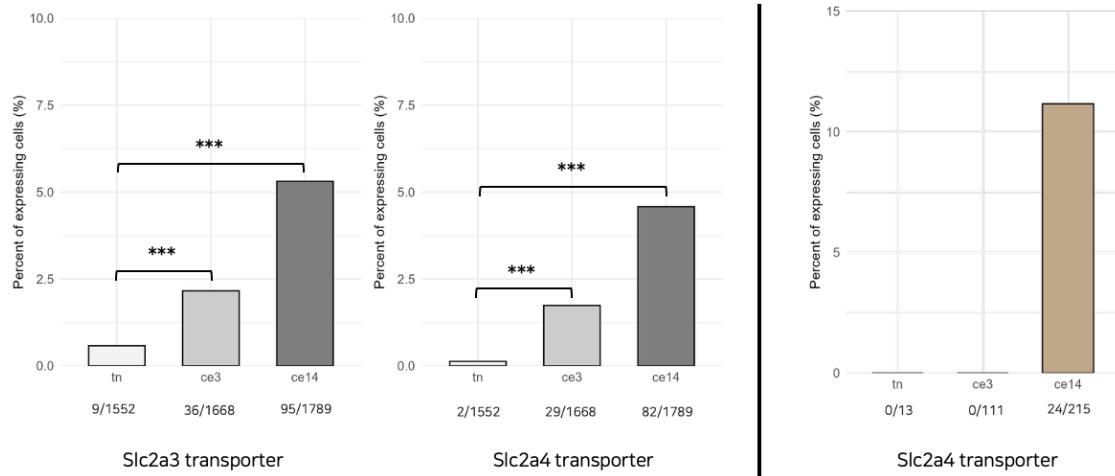
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Figure 8. Co-expression of DHA uptake and gamma-butyrobetaine synthesis genes across temperature conditions

Bar plots show the proportion of single cells co-expressing DHA transporters (*Slc2a3* and/or *Slc2a4*) and gamma-butyrobetaine synthesis genes under different temperature conditions. (A) Original dataset; (B) Validation dataset. White and beige adipocytes are indicated by white- and beige-colored bars, respectively. All increases were statistically significant (Fisher's exact test); *** indicates $p < 0.001$.

3.5 Observation of cells expressing the Bbox1 gene

To determine if gamma-butyrobetaine generated following DHA signaling is further converted to carnitine, we focused on Bbox1, which encodes gamma-butyrobetaine dioxygenase—the final enzyme in the carnitine biosynthesis pathway (Savic, Hodson et al. 2020). Since Bbox1-mediated catalysis represents the last step in the pathway, its expression serves as a direct indicator of a cell’s ability to complete carnitine synthesis. Although previous gene expression analyses (Figure 6A and B) examined both average expression levels and population frequencies, they did not clearly indicate whether Bbox1-expressing cells were increased under cold exposure. Therefore, we specifically assessed the presence and distribution of Bbox1-positive cells to determine whether this final step in carnitine biosynthesis could occur within a rare subset of adipocytes. Gamma-butyrobetaine is taken up by cells via the OCTN2 transporter, encoded by Slc22a5, and subsequently converted to carnitine through Bbox1-mediated catalysis (Fujita, Nakanishi et al. 2009).

Bbox1-expressing white adipocytes were nearly absent within white adipocyte clusters under room temperature conditions but became detectable upon cold exposure in the original dataset (Figure 9A). In the validation dataset, these cells were not observed under thermoneutrality or after 3 days of cold exposure, but appeared clearly after 14 days of cold challenge (Figure 9B). Although these cells accounted for only ~0.2% of the total white adipocyte population in both datasets, their consistent emergence across conditions

suggests that a rare subset of white adipocytes may possess the intrinsic capacity to complete carnitine synthesis through Bbox1-mediated catalysis (Figure 10). Furthermore, in the original dataset, we identified a small number of rare white adipocytes that co-expressed Bbox1 and Slc22a5, 0 out of 4 cells under RT, 7 out of 24 cells under CE4, and 10 out of 43 cells under CE7 (Figure 9C), indicating that rare white adipocytes may be capable of gamma-butyrobetaine uptake.

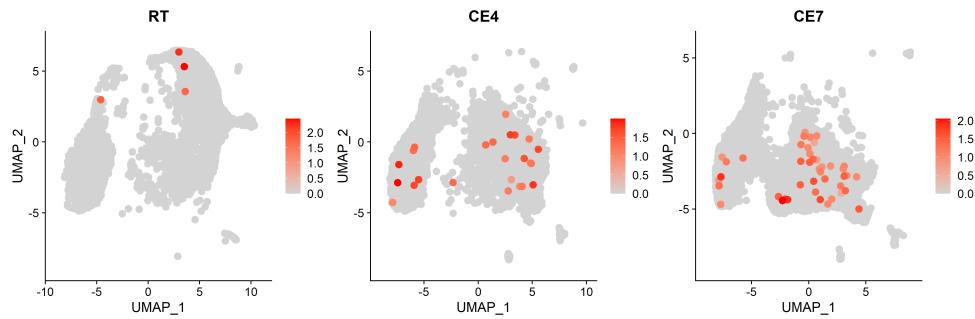
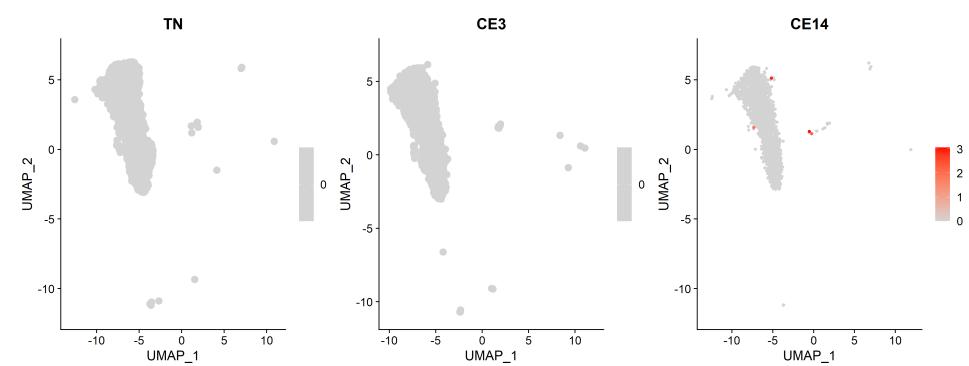
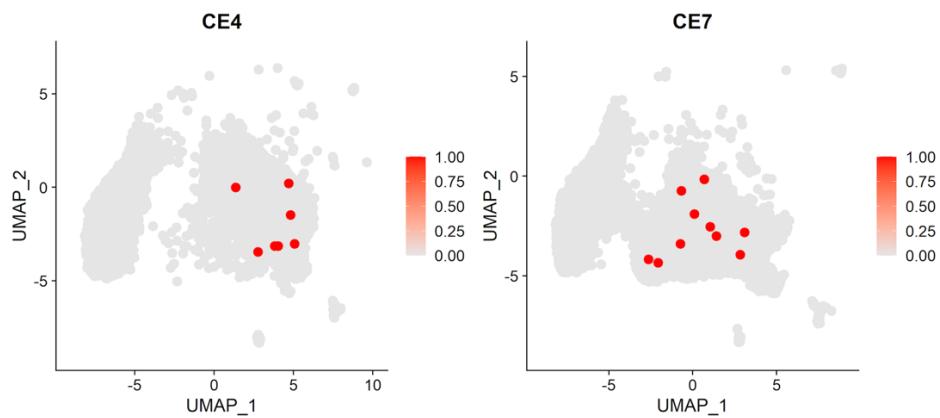
A**B****C**

Figure 9. Distribution of Bbox1-expressing cells white adipocytes

The spatial distribution of Bbox1-expressing cells was visualized using feature plots. These cells were primarily located within white adipocyte clusters, and expression intensity is also indicated. (A) Original dataset under three temperature conditions. (B) Validation dataset under similar conditions. (C) Cells co-expressing Bbox1 and Slc22a5 in the original dataset.

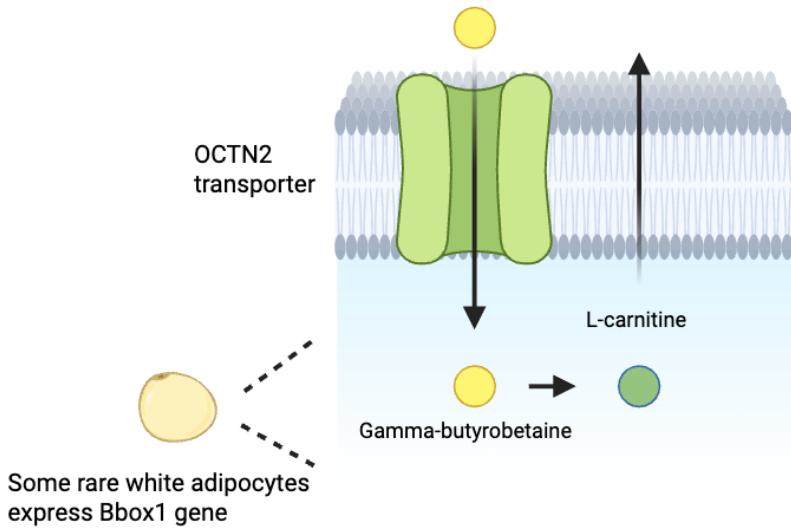


Figure 10. Diagram of rare white adipocytes completing carnitine synthesis

Illustration of gamma-butyrobetaine uptake via OCTN2 and its conversion to carnitine in rare white adipocytes. Created in BioRender.

3.6 Exploration of carnitine uptake in beige adipocytes

Previous studies suggests that carnitine contributes to thermogenesis in brown adipocytes, and our earlier analyses suggested that a rare subset of white adipocytes may complete carnitine synthesis from gamma-butyrobetaine. Given that beige adipocytes share thermogenic properties with brown adipocytes, we assumed that beige adipocytes have a role in taking up extracellular carnitine. Therefore, we examined beige adipocyte clusters for changes in the number of cells expressing the *Slc22a5* gene, which encodes carnitine uptake transporter (Longo, Frigeni et al. 2016), under cold conditions to assess their uptake capacity.

In both the original and validation datasets, we observed a substantial increase in the proportion of *Slc22a5*-positive beige adipocytes under cold conditions (Figure 11A and B). In the original dataset, the proportion rose from 5.3% (21/ 394) at RT to 43.7% (2270/5191) under CE4 and to 35% (1503/4294) under CE7. Similarly, in the original dataset, the proportion increased from 15.4% (2/13) at RT to 42.3% (47/111) under CE4 and to 54% (116/215) under CE7.

These results support the possibility that beige adipocytes import carnitine synthesized within inguinal white adipose tissue, potentially utilizing it to fuel thermogenesis (Figure 12). This cold-induced increase in *Slc22a5* expression suggests an enhanced carnitine uptake capacity in beige adipocytes. When considered alongside the presence of rare *Bbox1*-expressing white adipocytes capable of carnitine synthesis, our findings support a potential intercellular mechanism in which white adipocytes produce and supply carnitine

to beige adipocytes during cold adaptation.

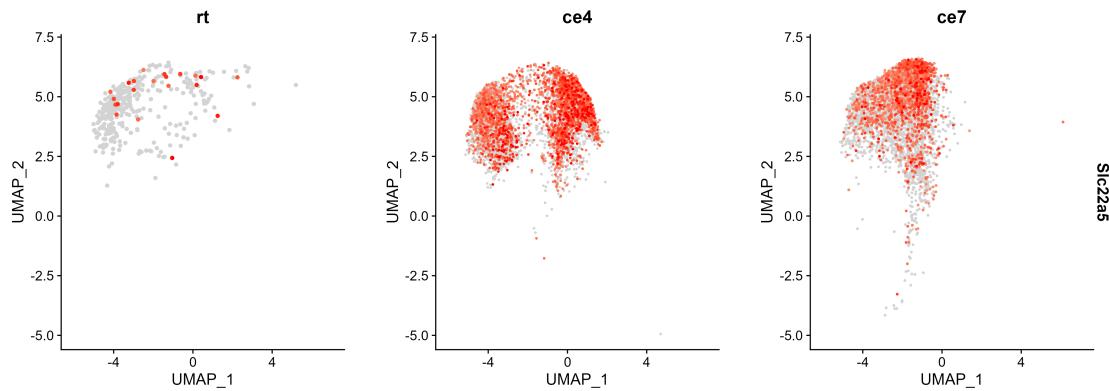
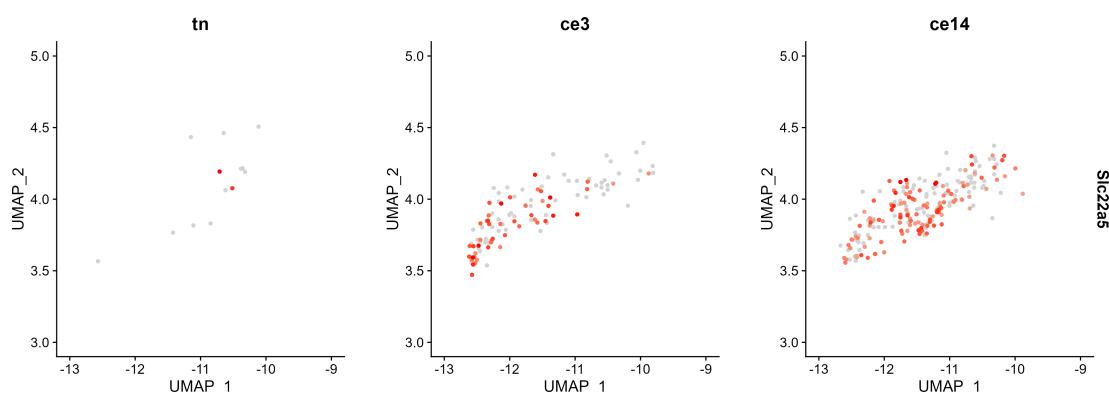
A**B**

Figure 11. Slc22a5 gene expression in beige adipocytes

Feature plots show the distribution and expression intensity of Slc22a5 under three temperature conditions. (A) Original dataset. (B) Validation dataset.

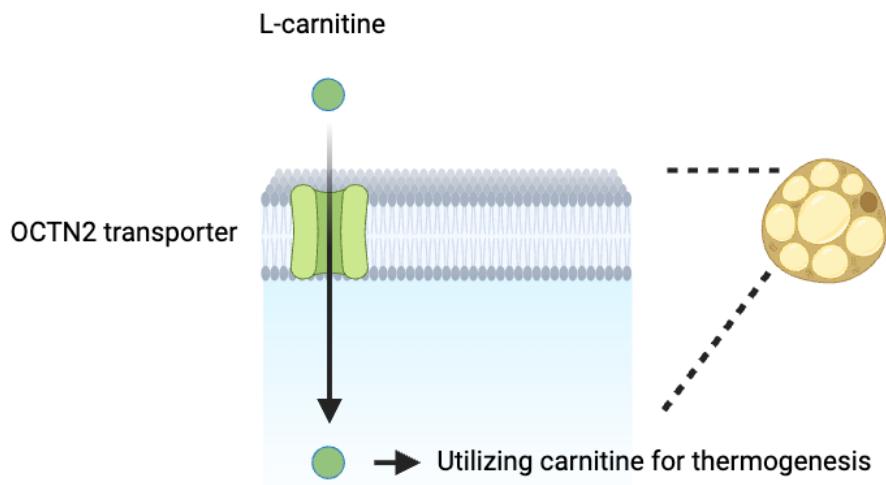


Figure 12. Carnitine uptake and utilization in beige adipocytes

Schematic illustration showing the proposed uptake of carnitine by beige adipocytes via the OCTN2 transporter encoded by Slc22a5 gene and its potential role in thermogenesis. Created in BioRender.

3.7 Profiling of carnitine related metabolite abundances by LC-MS

While scRNA-seq enables inference of metabolite-driven signaling based on gene expression patterns, it does not directly quantify metabolite levels. To complement this limitation, we measured the abundance of carnitine-related metabolites using liquid chromatography mass spectrometry (LC-MS) in inguinal white adipose tissue collected under corresponding environment conditions: thermoneutrality, room temperature, and severe cold. Among the detected metabolites, we focused on trimethyllysine, gamma-butyrobetaine, and carnitine—key intermediates in the carnitine biosynthesis pathway. This analysis was conducted to assess whether changes in their levels reflected transcriptional signals and reinforced their functional relevance.

LC-MS analysis revealed statistically significant increases in key metabolites: trimethyllysine, gamma-butyrobetaine, and carnitine under cold exposure (Figure 13). As a known precursor, the elevation of trimethyllysine, an upstream intermediate (Liepinsh, Kuka et al. 2021), suggests that the carnitine synthesis may become activated in response to cold exposure. Gamma-butyrobetaine, a direct intermediate immediately preceding carnitine in the pathway, also showed a marked increase. Although carnitine can be derived from dietary sources or circulation, the coordinated elevation of both gamma-butyrobetaine and carnitine implies that it is likely synthesized endogenously, rather than taken up exclusively. These findings support our hypothesis that carnitine is locally synthesized within the inguinal white adipose tissue potentially

by rare white adipocytes and subsequently utilized by beige adipocytes to support thermogenesis (Figure 14).

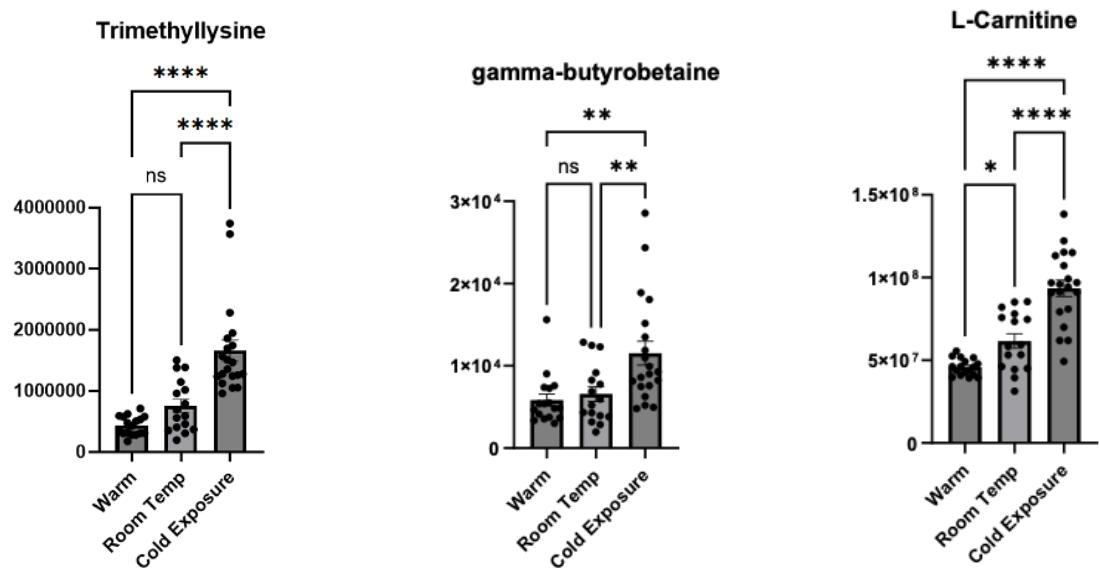


Figure 13. Temperature dependent changes in carnitine related metabolites

Abundances of trimethyllysine, gamma-butyrobetaine, and carnitine were measured by LC-MS under thermoneutrality, room temperature, and severe cold conditions. Data were analyzed and visualized using GraphPad Prism.

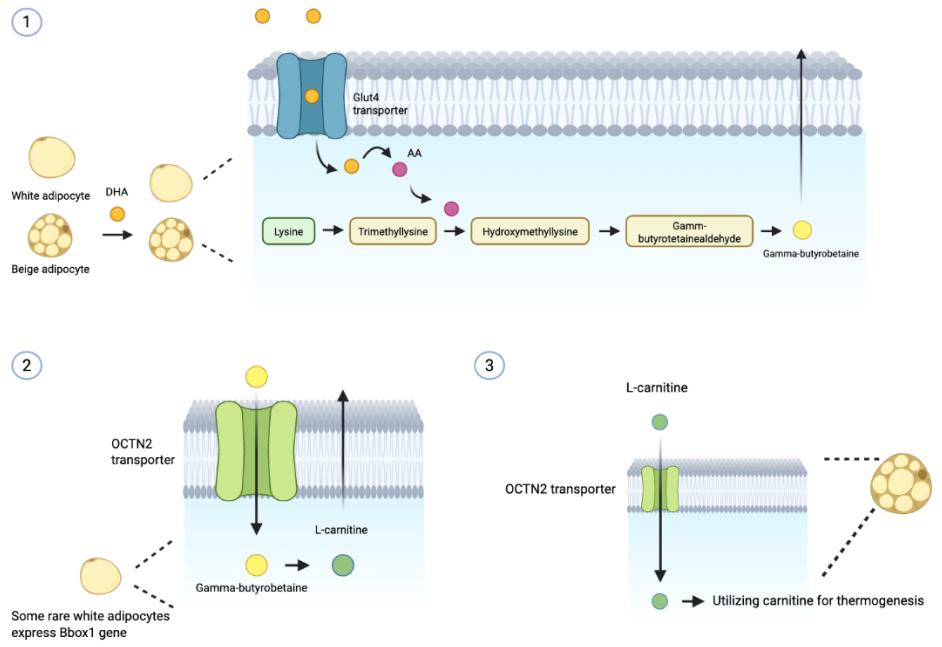


Figure 14. Summary of the proposed DHA-carnitine-thermogenesis pathway

Overview of our hypothesized mechanism linking DHA signaling to carnitine synthesis and its potential role in supporting thermogenesis DHA signals, carnitine synthesis and possible contribution to thermogenesis. Created in BioRender.

Discussion

In this study, we identified a potential metabolite-sensor communication mechanism involving dehydroascorbic acid (DHA) and the glucose transporters GLUT3 and GLUT4 (Rumsey, Kwon et al. 1997, Rumsey, Daruwala et al. 2000). Once transported into cells, DHA can be regenerated to AA (Wilson 2002), which plays a role in carnitine biosynthesis. Based on gene expression patterns, we propose that beige and white adipocytes contribute to the production of gamma-butyrobetaine, a carnitine precursor, and release it. Subsequently, a subset of rare white adipocytes may complete the carnitine synthesis pathway. To support transcriptomic inference, we validated the cold-induced increase in trimethyllysine, gamma-butyrobetaine, and carnitine using LC-MS, indicating activation of the carnitine synthesis pathway at the metabolite level.

Our research investigates the feasibility of inferring metabolite-based cell-to-cell signaling from transcriptomic data by leveraging metabolite-sensor interactions, in a manner analogous to protein ligand-receptor models. These protein-based models are fast, simple, and biologically interpretable, and are supported by many robust tools; however, they are inherently limited to protein-centric signaling and do not account for metabolite-sensor interactions. As both cell-to-cell metabolic activities are usually inferred indirectly from gene expression data, we sought to validate this prediction by assessing actual

metabolite abundance, enabling a more direct understanding of metabolic interactions between cells.

Through this analysis, we provided multiple lines of evidence suggesting that adipose tissue may be capable of autonomously engaging in carnitine-dependent thermogenesis. In particular, we identified a rare cell population with the potential to produce carnitine de novo. Validation using an independent dataset and LC-MS analysis further supports the existence of this cell population. Until now, carnitine-based biosynthesis has been primarily attributed to organs such as the liver. Our findings propose a novel paradigm where adipose tissue itself produces carnitine locally and utilizes it for thermogenesis, rather than depending on dietary sources.

Although our findings suggest the possibility of intercellular metabolite transfer based on gene expression patterns such as the potential movement of gamma-butyrobetaine to a rare subset of white adipocytes or the import of carnitine into beige adipocytes via transporters encoded by the Slc22a5 gene, the actual site of production and directionality of these metabolites remain unclear. To further clarify these interactions, spatial transcriptomics (Williams, Lee et al. 2022) could help reveal the spatial relationship between producer and recipient cells, and isotope-labeled metabolite tracing (Ruiz, Gelinas et al. 2015) would provide direct evidence for metabolite origin and intercellular transfer.

Beyond cold-induced thermogenic signaling, our metabolite-sensor communication framework can be applied to comparative analyses across

different physiological or environmental conditions. Even within the same adipose tissue, cellular responses may vary depending on external stimuli; for instance, dietary interventions such as comparing chow and high-fat diets can reveal how Western dietary patterns influence metabolic health and disease susceptibility. Furthermore, this framework is extendable to other metabolically active organs, including the heart, liver, kidney, and nervous system, where it may uncover novel signaling mechanisms involved in disease-associated metabolic regulation. For example, in the heart, altered metabolite signaling may contribute to the progression of heart failure (Lopaschuk, Karwi et al. 2021), while in the liver, metabolite-driven communication between hepatocytes and immune cells may play a role in insulin resistance and the development of fatty liver disease (Lim, Taskinen et al. 2019). These applications of our framework could provide broader insight into how metabolic communication is altered in diverse physiological and pathological contexts, including obesity, cardiovascular disorder, and metabolic syndrome.

References

1. Alberts, B. J., A.; Lewis, J (2002). "Molecular Biology of the Cell. 4th edition." New York: Garland Science.
2. Allison, J. R. U., W.; Carrie, M.; Jacqueline, M (2020). "Adipose Tissue: Physiology to Metabolic Dysfunction." National Institutes of Health (NIH).
3. Almet, A. A., et al. (2021). "The landscape of cell-cell communication through single-cell transcriptomics." *Curr Opin Syst Biol* 26: 12–23.
4. Baker, S. A. and J. Rutter (2023). "Metabolites as signalling molecules." *Nature Reviews Molecular Cell Biology* 24(5): 355–374.
5. Fujita, M., et al. (2009). "Hepatic uptake of gamma-butyrobetaine, a precursor of carnitine biosynthesis, in rats." *Am J Physiol Gastrointest Liver Physiol* 297(4): G681–686.
6. Hagberg, C. E. and K. L. Spalding (2024). "White adipocyte dysfunction and obesity-associated pathologies in humans." *Nat Rev Mol Cell Biol* 25(4): 270–289.
7. Hao, Y., et al. (2024). "Dictionary learning for integrative, multimodal and scalable single-cell analysis." *Nat Biotechnol* 42(2): 293–304.
8. Haque, A., et al. (2017). "A practical guide to single-cell RNA-sequencing for biomedical research and clinical applications." *Genome Med* 9(1): 75.

9. Holman, C. D., et al. (2024). "Aging impairs cold-induced beige adipogenesis and adipocyte metabolic reprogramming." *Elife* 12.
10. Ikeda, K., et al. (2018). "The Common and Distinct Features of Brown and Beige Adipocytes." *Trends Endocrinol Metab* 29(3): 191–200.
11. Jin, S., et al. (2021). "Inference and analysis of cell-cell communication using CellChat." *Nat Commun* 12(1): 1088.
12. Kershaw, E. E. and J. S. Flier (2004). "Adipose tissue as an endocrine organ." *Journal of Clinical Endocrinology & Metabolism* 89(6): 2548–2556.
13. Kim, H. Y. (2017). "Statistical notes for clinical researchers: Chi-squared test and Fisher's exact test." *Restor Dent Endod* 42(2): 152–155.
14. Liepinsh, E., et al. (2021). "Low cardiac content of long-chain acylcarnitines in TMLHE knockout mice prevents ischaemia-reperfusion-induced mitochondrial and cardiac damage." *Free Radic Biol Med* 177: 370–380.
15. Lim, S., et al. (2019). "Crosstalk between nonalcoholic fatty liver disease and cardiometabolic syndrome." *Obes Rev* 20(4): 599–611.
16. Longo, N., et al. (2016). "Carnitine transport and fatty acid oxidation." *Biochim Biophys Acta* 1863(10): 2422–2435.
17. Lopaschuk, G. D., et al. (2021). "Cardiac Energy Metabolism in Heart Failure." *Circ Res* 128(10): 1487–1513.
18. Martinez-Reyes, I. and N. S. Chandel (2020). "Mitochondrial TCA cycle metabolites control physiology and disease." *Nat Commun* 11(1): 102.

19. Ozaki, K. S., T.; Tsuji, N. et al (2011). "Carnitine is necessary to maintain the phenotype and function of brown adipose tissue." *Laboratory Investigation*.
20. Rines, A. K., et al. (2015). "Adenosine activates thermogenic adipocytes." *Cell Res* 25(2): 155–156.
21. Ruiz, M., et al. (2015). "Metabolic Tracing Using Stable Isotope-Labeled Substrates and Mass Spectrometry in the Perfused Mouse Heart." *Methods Enzymol* 561: 107–147.
22. Rumsey, S. C., et al. (2000). "Dehydroascorbic acid transport by GLUT4 in Xenopus oocytes and isolated rat adipocytes." *J Biol Chem* 275(36): 28246–28253.
23. Rumsey, S. C., et al. (1997). "Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid." *J Biol Chem* 272(30): 18982–18989.
24. Savic, D., et al. (2020). "The Importance of the Fatty Acid Transporter L-Carnitine in Non-Alcoholic Fatty Liver Disease (NAFLD)." *Nutrients* 12(8).
25. Saxton, R. A. and D. M. Sabatini (2017). "mTOR Signaling in Growth, Metabolism, and Disease." *Cell* 168(6): 960–976.
26. Su, J., et al. (2024). "Cell-cell communication: new insights and clinical implications." *Signal Transduct Target Ther* 9(1): 196.
27. Wang, T., et al. (2024). "Single-nucleus transcriptomics identifies separate classes of UCP1 and futile cycle adipocytes." *Cell Metab* 36(9): 2130–2145 e2137.
28. Wang, Z., et al. (2010). "Specific metabolic rates of major organs and tissues across adulthood: evaluation by mechanistic model of resting energy expenditure." *Am J Clin Nutr* 92(6): 1369–1377.

29. Williams, C. G., et al. (2022). "An introduction to spatial transcriptomics for biomedical research." *Genome Med* 14(1): 68.
30. Wilson, J. X. (2002). "The physiological role of dehydroascorbic acid." *FEBS Lett* 527(1-3): 5-9.
31. Wolf, F. A., et al. (2018). "SCANPY: large-scale single-cell gene expression data analysis." *Genome Biol* 19(1): 15.
32. Ying, Z., et al. (2023). "Role of thermogenic adipose tissue in lipid metabolism and atherosclerotic cardiovascular disease: lessons from studies in mice and humans." *Cardiovasc Res* 119(4): 905-918.
33. Yook, J. S., et al. (2021). "Dietary Iron Deficiency Modulates Adipocyte Iron Homeostasis, Adaptive Thermogenesis, and Obesity in C57BL/6 Mice." *J Nutr* 151(10): 2967-2975.
34. Zheng, R. Z., Y; Tsuji, T.; Gao, X.; Shamsi, F; Wagner, A.; Yosef, N.; Chen, H.; Zhang, L.; Tseng, Y.; Chen, K (2022). "MEBOCOST: Metabolite-mediated Cell Communication Modeling by Singl cell Transcriptome." *bioRxiv*.

논문요약

단일세포 RNA 시퀀싱을 통한 지방조직 내 대사성

세포신호 분석

방은규

생명과학과

성균관대학교

단일세포 RNA 시퀀싱 (Single-cell RNA sequencing, scRNA-seq)은 개별 세포 수준에서 전사체 (transcriptome)를 분석을 가능하게 하여 세포 간 이질성을 정밀하게 관찰할 수 있는 강력한 도구로 자리매김하고 있다. 최근 scRNA-seq 기술의 발전은 세포 간 신호 전달 (cell-to-cell communication) 연구를 가속화하며, 생체 항상성을 유지하는 데 핵심적인 신호전달 기작에 대한 이해를 넓히는 데 기여하고 있다. 신호전달은 주로 단백질과 대사산물 (metabolite)에 의해 조절되며, 전통적으로 단백질 기반의 상호작용이 활발히 연구되어온 반면, 대사산물 기반의 신호전달에 대한 연구는 상대적으로 부족하다.

본 연구에서는 이러한 연구 공백을 해소하고자, 단일세포 전사체 데이터를 기반으로 대사효소 및 센서 단백질의 발현을 분석하여 대사산물 기반의 세포 간 신호전달 네트워크를 추론할 수 있는 계산 알고리즘인 MEBOCOST 를 활용하였다.

이를 통해 대사산물-센서 기반의 송신세포 (sender cell) 및 수신세포 (target cell)를 식별하고, 대사산물이 매개하는 새로운 신호전달 경로를 탐색하였다.

연구모델로는 전신 에너지 항상성에 중요한 역할을 하는 대사 활성 조직인 지방조직 (adipose tissue)을 선정하였으며, 특히 냉자극 (cold exposure)에 의해 유도되는 열발생 (thermogenesis) 상태에서의 신호전달 양상을 분석하였다. MEBOCOST 분석과 후속 유전자 발현 분석 결과, 서혜부 백색 지방조직 (inguinal white adipose tissue)에서 기존에 보고되지 않았던 dehydroascorbic acid (DHA)를 중심으로 하는 새로운 신호전달 경로가 관찰되었다. 특히 beige 지방세포와 white 지방세포 모두가 carnitine 생합성에 기여하며, 이는 DHA 기반 신호전달에 의해 조절 될 가능성이 있는 것으로 나타났다.

본 연구는 대사산물을 매개로 한 세포 간 신호전달을 정량적, 체계적으로 분석할 수 있는 전략을 제시하며, 향후 다른 대사 활성 조직이나 질환 상황에서도 적용 가능한 분석 프레임워크로 확장될 수 있을 것으로 기대된다.