Identifying potential regulators of JAGGED1 expression in portal mesenchymal cells.

Teppei Nishino
University of Tsukuba: Tsukuba Daigaku
Masaharu Yoshihara (yoshihara.masahar.ly@alumni.tsukuba.ac.jp)
University of Tsukuba: Tsukuba Daigaku https://orcid.org/0000-0002-0212-0909
Takahiro Nakayama
University of Tsukuba: Tsukuba Daigaku
Takaho Tsuchiya
University of Tsukuba: Tsukuba Daigaku
Saeko Tahara
University of Tsukuba: Tsukuba Daigaku
Haruka Ozaki
University of Tsukuba: Tsukuba Daigaku
Satoru Takahashi
University of Tsukuba: Tsukuba Daigaku

Research Article

Keywords: Delta-Notch signaling pathway, DoRothEA, EGR1, intrahepatic biliary development, regulon evaluations, SLUG, single cell RNA-seq, smooth muscle actin, SOX2, transcription factors

Posted Date: February 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1210544/v1

Abstract

Objective: Portal mesenchymal cells induces the epithelial differentiation of the bile ducts in the developing liver via one of the Delta-Notch signaling components, JAGGED1. Although this differential induction is crucial for normal liver physiology as its genetic disorder (Alagille syndrome) causes jaundice, the molecular mechanism behind JAGGED1 expression remain unknown. Here, we searched for upstream regulatory transcription factors of JAGGED1 using an integrated bioinformatics method.

Results: According to DoRothEA database, which integrated multiple lines of evidence on the relation between transcription factors and its downstream target genes, three transcription factors were predicted to be upstream of JAGGED1: SLUG, SOX2 and EGR1. Among these, SLUG and EGR1 were enriched in ACTA2 expressing portal mesenchymal cells in the previously reported two human fetal liver single cell RNA-seq data. JAGGED1 expressing portal mesenchymal cells tended to express of SLUG rather than EGR1, supporting that SLUG induced JAGGED1 expression. Together with the higher confidentiality of SLUG (DoRothEA level A) over EGR1 (DoRothEA level D), we concluded that SLUG was one of the most important candidate transcription factors upstream of JAGGED1. These results would add mechanistic insights into developmental biology on how portal mesenchymal cells supports biliary development in the liver.

Introduction

Identification of the upstream regulators of key genes is one of the central issues in developmental biology. In the field of the bile duct formation in the liver, one of the Delta-Notch signaling components, JAGGED1, is crucial as understood by jaundice in the patients with its genetic abnormalities (Alagille syndrome) [1,2]. One of the major complications of this disease is abnormalities in the bile ducts in the liver, often requiring liver transplantation. JAGGED1 is expressed in portal mesenchymal cells that are characterized by smooth muscle actin-associated genes such as TAGLN [3] and ACTA2 [4]. In an experimental point of view, TAGLN-Cre-mediated JAGGED1 deletion in portal mesenchymal cells caused significant jaundice [3], suggesting that portal mesenchymal cells express JAGGED1 to induce epithelial differentiation that occurs in their periphery between mouse embryonic days 13.5 (E13.5) and E18.5 [5]. However, the molecular mechanism underlying the induction of JAGGED1 expression in portal mesenchymal cells remains unknown. As portal mesenchymal cells occupy small percentage (less than 10%) of the total liver cells (discussed later), we considered that it would be difficult to identify potential upstream transcription factors using conventional biochemical methods.

Given this, we went on to identify the upstream transcription factor regulating the induction of JAGGED1 expression in portal mesenchymal cells, using an established bioinformatics database called DoRothEA [6]. DoRothEA uses several lines of data to generate a table of combinations of specific transcription factors and their downstream targets (regulons) with the confidentiality of these combinations scored from A (highest confidence) to E (lowest confidence), depending on the amount of evidence supporting these interactions. This means that a DoRothEA level A interaction is supported by at least two curated

sources. We used this database to identify potential upstream transcription factors regulating JAGGED1 expression. We then examined the validity of this results by analyzing two sets of previously reported human fetal liver single cell RNA-seq data [7]. This strategy of narrowing down the potential transcription factors would provide realistic insights into regulation of a developmentary important gene component, JAGGED1.

Methods

We used R (R version 3.6.3; https://www.R-project.org/) to refer to DoRothEA.

We used the raw FASTQ files of single-cell RNA sequencing data from human fetal livers at Carnegie Stage (CS) 20 (GSM3906001) and 23 (GSM3906002), which correspond to E14.5 and E16, respectively. Both datasets were retrieved from the NCBI Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). We used CellRanger (v5.0.1) [8] to align sequencing reads in the FASTQ files to human reference (GRCh38-2020-A, 10x Genomics) and creating count matrix for following analysis. We used Scanpy (v1.7.0) [9] for basic filtering and comparing the enrichment of specific genes between one cluster and the others. Cells with high percentages of mitochondrial (> 0.1) or hemoglobin gene (> 0.1) expression and cells with a low percentage of ribosomal gene expression (< 0.05) were excluded from subsequent analysis. Any doublets identified by Scrublet (v0.2.3) were also excluded. Then highly variable genes (n = 3,019 and 2,603 for CS20 and 23 datasets, respectively) across the single cell datasets (n = 7,028 and 8,020 for CS20 and 23 datasets, respectively) were identified using the “sc.pp.highly_variable_genes” function, and then, subjected to dimension reduction with principal component analysis (“sc.tl.pca” function) and UMAP (“sc.tl.umap” function) before clustering (Leiden method). Single-cell RNA sequencing analysis was completed in Python 3.6.13 in an Ubuntu 20.04 LTS environment. We examined differences in gene expression within these clusters using the Mann-Whitney U test, with these outcomes described as FDR-adjusted p values in Results section.

Results

SLUG, SOX2 and EGR1 as potential regulators for JAGGED1

We first used DoRothEA to identify several potential regulators of JAGGED1 in portal mesenchymal cells and focused on SLUG (also known as SNAI2), SOX2, and EGR1, which were listed in DoRothEA database for their association with JAGGED1 (levels A, B and D, respectively) [6]. There were no candidate transcription factors with DoRothEA levels A or B other than SLUG and SOX2. EGR1 may be important because it was expressed in portal mesenchymal cells (discussed later) whereas the other transcription factors of DoRothEA level C or D, which were listed in Additional File 1, were not enriched in portal mesenchymal cells in either CS20 or 23 datasets.

Identification of ACTA2 expressing portal mesenchymal cells

Next, we searched for TAGLN expressing portal mesenchymal cells in previously reported single-cell RNA-seq data from human fetal liver tissues of CS20 and 23 datasets as described in Method section. Unfortunately, those cells occupied only 3.54% and 1.11% of total cells of CS20 and 23 datasets, respectively. Therefore, we alternatively searched for ACTA2 expressing portal mesenchymal cells in CS20 and 23 datasets. In contrast to TAGLN, ACTA2 expressing cells occupied 5.92% and 5.76% of total cells of CS20 and 23 datasets, respectively, and we determined to use ACTA2 as portal mesenchymal cell marker gene. For CS20 dataset, we conducted cell clustering as described in Method section and that evaluation produced 21 clusters (Figure 1a), one of which (cluster #6) contained 466 cells (6.63% of total cells) with increased ACTA2 ($p = 8.21 \times 10^{-24}$) expression (Figure 1b), identifying them as the portal mesenchymal cells. As expected, this cell population expressed a relatively high level of JAGGED1 (log fold-change = 2.30, p = 0.439) (Figure 1c). For CS 23 dataset, these evaluations produced 19 clusters (Figure 1d), one of which (cluster #15) contained 129 cells (4.96% of total cells) with increased ACTA2 ($p = 1.80 \times 10^{-28}$) expression (Figure 1e), identifying them as the portal mesenchymal cells. Indeed, this cell population expressed a significantly high level of JAGGED1 (p = 0.0384) (Figure 1f).

Evaluation the validity of SLUG, SOX2 and EGR1

Then, we examined the expression of SLUG, SOX2 and EGR1 in portal mesenchymal cells (cluster #6 and #15 of CS20 and 23 datasets, respectively). For CS20 dataset, the cells in cluster #6 abundantly expressed SLUG ($p = 2.08 \times 10^{-51}$) (Figure 2a) and EGR1 ($p = 5.91 \times 10^{-22}$) (Figure2c), but not SOX2 (p = 1.00) (Figure 2b). We further evaluated the expression of JAGGED1, SLUG and EGR1 in the cells of cluster #6 of CS20 dataset. This evaluation showed that 63.6% JAGGED1 expressing cells also expressed SLUG (7 out of the 11 cells), while 13.2% JAGGED1 non-expressing cells expressed SLUG (60 out of the 455 cells) ($p = 1.95 \times 10^{-4}$, Fisher's exact test) (Figure 2d). Additionally, although 63.6% JAGGED1 expressing cells also expressed EGR1 (7 out of the 11 cells), only 16% JAGGED1 non-expressing cells expressed EGR1 (73 out of the 455 cells) ($p = 6.37 \times 10^{-4}$, Fisher's exact test), and hence, we considered that both SLUG and EGR1 remained as potential regulators (Figure 2e).

For CS23 dataset, the cells in cluster #15 abundantly expressed SLUG ($p = 2.29 \times 10^{-13}$) (Figure 2f) and EGR1 ($p = 9.51 \times 10^{-6}$) (Figure 2h), but not SOX2 (p = 1.00) (Figure 2g). We further evaluated the expression of JAGGED1, SLUG and EGR1 in the cells of cluster #15. This evaluation showed that 50.0% JAGGED1 expressing cells also expressed SLUG (3 out of the 6 cells), while only 22.8% JAGGED1 non-expressing cells expressed SLUG (28 out of the 123 cells) (p = 0.150, Fisher's exact test) (Figure 2i). In contrast, although 16.7% JAGGED1 expressing cells also expressed EGR1 (1 of the 6 cells), as much as 28.5% JAGGED1 non-expressing cells expressed EGR1 (35 of the 88 cells) (p = 1.00, Fisher's exact test) (Figure 2j). Therefore, together with high confidentiality (DoRothEA level A), we concluded that SLUG is preferable candidate regulator of JAGGED1 expression in portal mesenchymal cells.

Discussion

This study was designed to identify the upstream transcription factor regulating JAGGED1 expression in portal mesenchymal cells. Our evaluations found SLUG to be a central candidate for this regulatory role as this protein is strongly expressed during the time frame associated with epithelial differentiation into portal mesenchymal cells. We considered that insignificant JAGGED1 expression in portal mesenchymal cells at CS20 was because this time point was relatively early to allow the expression of the upstream regulator, SLUG, rather than JAGGED1.

SLUG reportedly acts as a transcriptional suppressor, as previously observed during the downregulation of E-cadherin expression in breast cancer [10]. However, result of the biochemical analysis of the induction of fatty acid synthase in the liver suggests that SLUG can act as an activator [11]. Additionally, an *in vitro* study using a human breast cancer cell line (MCF7) showed that SLUG overexpression or siRNA-mediated suppression leads to increased or decreased JAGGED1 expression, respectively, suggesting that SLUG expression is positively correlated with JAGGED1 expression in this cell line [12]. Although this observation, along with the absence of SLUG binding sites in the JAGGED1 promoter region according to DoRothEA [6], imply that SLUG does not directly regulate JAGGED1 expression, we propose that SLUG is located upstream of JAGGED1.

EGR1 is also a potential regulator, enriched in mesenchymal cells (cluster #6 of CS20 dataset and cluster #15 of CS23 dataset). This transcription factor has been reported to induce JAGGED1 expression in *Leishmania donovani*-infected bone marrow macrophages [13]. However, owing to its low confidentiality (DoRothEA level D) and scarce expression in JAGGED1 expressing portal mesenchymal cells in CS23 dataset, we consider SLUG as a potential regulator over EGR1.

Conclusion

SLUG is the candidate regulator of JAGGED1 in portal mesenchymal cells.

Limitations

Firstly, further *in vivo* analysis is necessary to examine the biochemical properties of the portal mesenchymal cells to determine the relationships between transcription factors and their regulons. Secondly, this study does not suggest that SLUG is the sole candidate for the upstream regulation of JAGGED1 expression because we only evaluated transcription factors according to DoRothEA. Thirdly, four transcription factors (ESR1, HNB1B, PRDM14, and TFAP2C) were not examined for its enrichment because they were not differentially expressed genes in either CS20 or 23 datasets. Finally, we note that the molecular signature of portal mesenchymal cells is not fully characterized, and hence, other transcription factors may be listed as potential regulators when we use other marker genes for portal mesenchymal cells. However, we consider our work is significant in that this is the first report on the molecular regulation of JAGGED1 expression in the portal mesenchymal cells, which is important for normal liver physiology.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Codes of the single-cell RNA sequencing analyses are available from our GitHub repository (https://github.com/Teppei-Nishino/Slug).

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by JSPS KAKENHI Grant Number JP21J20959. MY was also supported by the Japan Society for the Promotion of Science through Research Fellowships for Young Scientists (DC1).

Author's contributions

Teppei Nishino - Data Curation, Formal Analysis, Investigation, Software, Visualization, Writing – original draft, MY: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Takahiro Nakayama - Writing – review & editing, TT - Conceptualization, Methodology, Writing – review & editing, Saeko Tahara - Writing – review & editing, HO - Writing – review & editing, Satoru Takahashi - Project administration, Resources.

Acknowledgements

The authors would like to thank the members of the Department of Anatomy and Embryology from the Faculty of Medicine at the University of Tsukuba, for their helpful discussions. We would also like to thank Editage (www.editage.com) for their assistance with the English language editing of this manuscript.

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Figures

Figure 1

Identification of portal mesenchymal cells in human fetal liver single cell RNA-seq data (a)UMAP plot of CS20 human fetal liver single cell RNA-seq data. (b) Cluster #6 in (a) was enriched for ACTA2 and contained portal mesenchymal cells (arrowhead). (c) Cluster #6 contained JAGGED1 expressing cells (arrowhead). (d) UMAP plot of CS23 human fetal liver single cell RNA-seq data. (e) Cluster #15 in (d) was enriched for ACTA2 and contained portal mesenchymal cells (arrowhead). (f) Cluster #6 contained JAGGED1 expressing cells (arrowhead).

Figure 2

Examination of enrichment of potential regulators in portal mesenchymal cells. (a) SLUG was enriched in cluster #6 of CS20 dataset (FDR-adjusted p value = 2.08×10^{-51}). (b) SOX2 was not enriched in cluster #6 of CS20 dataset (FDR-adjusted p value = 1.00). (c) EGR1 was enriched in cluster #6 of CS20 dataset (FDR-adjusted p value = 5.91×10^{-22}). (d) Comparison of JAGGED1 or SLUG expression in the cells of cluster #6 of CS20 dataset as evaluated by Fisher's exact test. Note that percentage of SLUG expressing cells were significantly higher in JAGGED1 expressing cells than in JAGGED1 non-expressing cells ($p = 1.95 \times 10^{-4}$). (e) Comparison of JAGGED1 or EGR1 expression in the cells of cluster #6 of CS20 dataset as evaluated by Fisher's exact test. Note that percentage of EGR1 expressing cells were significantly higher in JAGGED1 expressing cells than in JAGGED1 non-expressing cells ($p = 6.37 \times 10^{-4}$). (f) SLUG was enriched in cluster #15 of CS23 dataset (FDR-adjusted p value = 2.29×10^{-13}). (g) SOX2 was not enriched in cluster #15 of CS23 dataset (FDR-adjusted p value = 1). (h) EGR1 was enriched in cluster #15 of CS23 dataset (FDR-adjusted p value = 9.51×10^{-6}). (i) Comparison of JAGGED1 or SLUG expression in the cells of cluster #15 of CS23 dataset as evaluated by Fisher's exact test. Note that percentage of SLUG expressing cells were high in JAGGED1 expressing cells than in JAGGED1 non-expressing cells although there was no statistical significance ($p = 0.150$). (j) Comparison of JAGGED1 or EGR1 expression in the cells in cluster #15 of CS23 dataset as evaluated by Fisher's exact test. Note that percentage of EGR1 expressing cells were lower in JAGGED1 expressing cells than in JAGGED1 non-expressing cells although there was no statistical significance ($p = 1.00$).

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