Development of Technology for Quality Evaluation of Human Pluripotent Stem Cells by Metabolome Analysis

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Abstract

To establish a technology for quality evaluation of pluripotent stem cells with metabolome analysis, we optimized sample preparation methods and developed an analytical method for gas chromatography/mass spectrometry-based metabolomics. This method enables us to quantify various species of metabolites including those associated with the glycolysis and citric acid cycles with high reproducibility. Comparative metabolomics showed a clear difference between undifferentiated and differentiated states of human embryonic stem cell lines. Thus, we were able to confirm the applicability of metabolome analysis to the quality evaluation of pluripotent stem cells.

Keyword:GC/MS, Human embryonic stem cells, Human pluripotent stem cells, Metabolome analysis

1. Introduction

Pluripotent stem cells have an unlimited self-renewing capacity, and can differentiate into any cell type in the body[1)]. They consequently hold great promise as a source of cells for applications in regenerative medicine and drug discovery[2), 3)]. For these applications, it is essential that technology is developed for the mass production of high quality pluripotent stem cells. We are participating in the New Energy and Industrial Technology Development Organization (NEDO) project "Fundamental Technology for Promoting Industrial Application of Human Stem Cells" to establish a technology for the quality evaluation of pluripotent stem cells using metabolome analysis. Metabolomics is thought to be directly connected to the phenotype of an organism[4)], and is thus expected to provide extremely valuable information for the development of biomarkers for the quality evaluation of pluripotent stem cells. Metabolome analysis of pluripotent stems cells is also anticipated to become increasingly important in the future since metabolomics complements other omics datasets. However, compared to other omics datasets, there are only several examples of metabolomics being used in the study of pluripotent stem cells[5)-9)]. The development of a metabolome analysis method suitable for pluripotent stem cells is an important part of accumulating valuable information in the form of stem cell metabolome datasets. Accordingly, we have developed an analytical method for analysis of the metabolome of pluripotent stem cells. GC-MS-based metabolomics studies have been conducted since the beginning of metabolomics and remain a powerful technology for metabolomics. The reasons for this include the existence of many databases of MS spectra that allow for easy interpretation of MS spectra obtained experimentally, and the high resolution separation performance of GC useful for the separation of complex biological mixtures.

We have developed a method for metabolome analysis of pluripotent stem cells with excellent comprehensiveness and reproducibility and have confirmed this method can distinguish between undifferentiated and differentiated human embryonic stem (ES) cells. The results are described in this article.

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2. Development of a Method of Metabolome Analysis of Pluripotent Stem Cells

2.1 Optimization of the Pretreatment Method

The first step in metabolome analysis of pluripotent stem cells is a process of detachment and recovery of cells from the culture dish. To obtain useful information on the intracellular metabolome, it is necessary to remove the culture medium and then rapidly stop all cellular enzymatic activity. Adherent cell cultures, including pluripotent stem cells, are normally recovered using enzymatic treatment such as with trypsin. However, this conventional method requires a complicated process that includes centrifugation and cell washing. The intracellular metabolome may also alter during the treatment process since no nutrient is provided to the cells. Consequently, we carried out cell recovery using the method of Teng et al.[10)] (Fig. 1) that consists of removing culture medium with an aspirator, washing cells twice in cold physiological saline solution, immediately adding methanol solution then using a cell lifter to perform physical detachment and recovery of cells (hereinafter referred to as "the quenching method"). Although there are other methods of stopping intracellular metabolism (see review by Léon et al.[11)] and its references), the quenching method was used for reasons of convenience: it is able not only to stop metabolic activity, but also to simultaneously extract intracellular metabolites. To confirm the significance of the cell recovery process, we extracted metabolites from cell samples recovered using the quenching method and from cell samples recovered using a trypsin method and analyzed them using GC-MS. The signal intensity of some metabolites was decreased in cell samples recovered using the trypsin method (Fig. 2A). For example, the signal intensity of glucose and glutamine is reduced by a factor of 50 times or more in cell samples recovered using the trypsin method compared to cell samples recovered using the quenching method (Fig. 2B). These results suggest that the intracellular metabolome changed substantially during trypsin treatment, and show cell recovery is a very important step in metabolome analysis in cultured cells.

Next, we studied methods of extracting metabolites. Sellick et al. [12)] reported extracting a wide range of metabolites with good

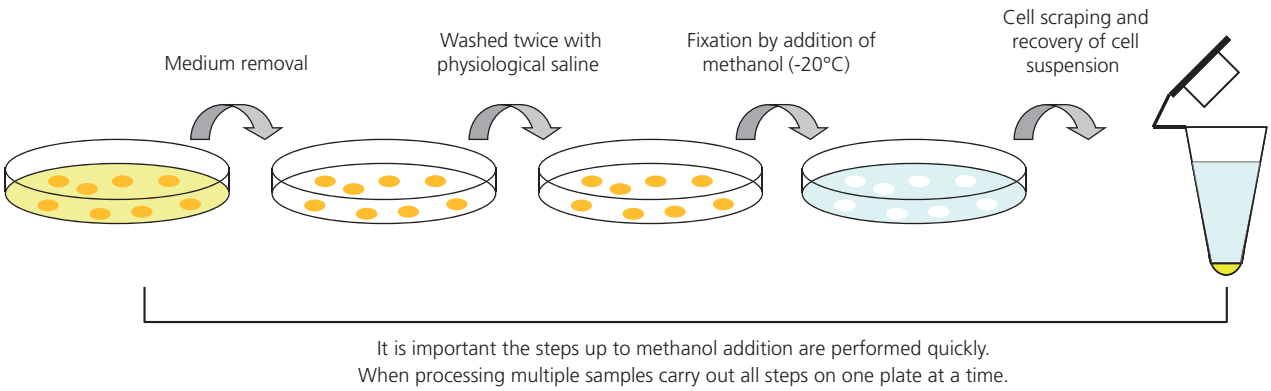


Fig. 1 Sample preparation of human pluripotent stem cells for metabolome analysis

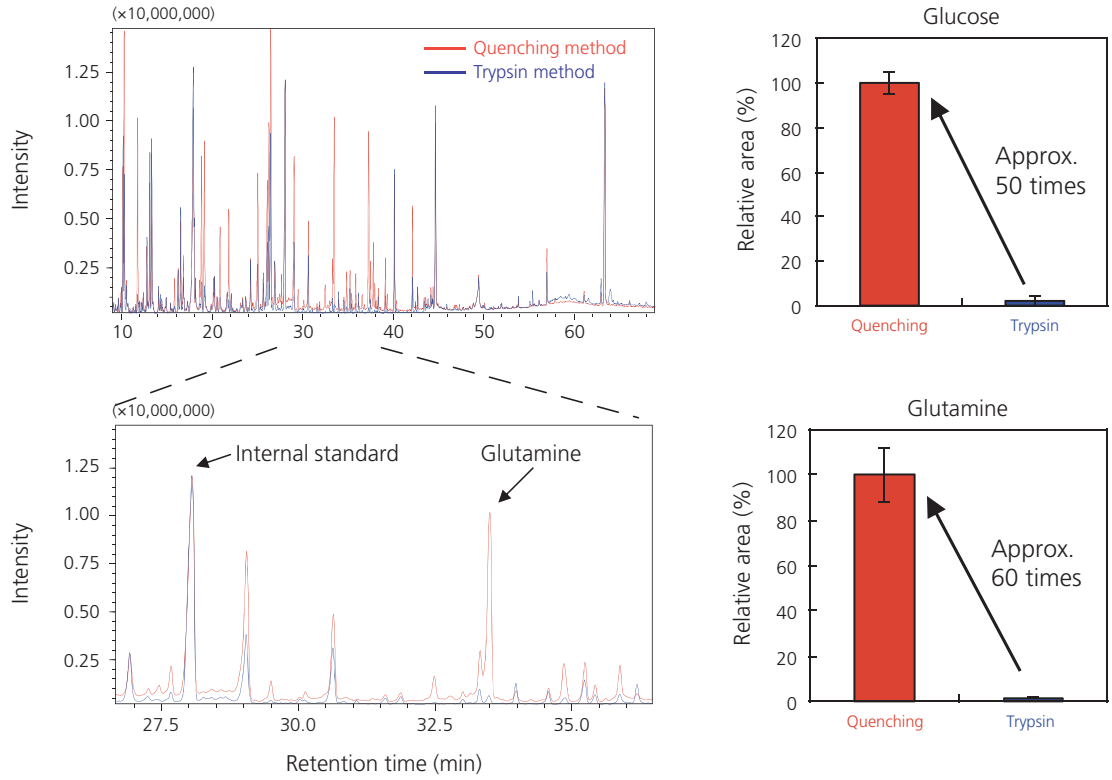


Fig. 2 Influence of sample preparation steps on metabolome analysis data

reproducibility using methanol extraction followed by a single extraction with water (hereinafter referred to as "the MeOH-H_2O method"). We investigated methanol extraction at various concentrations (from 60% to 100%), as well as the MeOH-H_2O method. Results showed no substantial change in the total ion current chromatogram (TIC) obtained between each of the extraction conditions investigated (results not shown). A comparison of the measured metabolite areas also confirmed no substantial difference in results between each of the extraction conditions for organic acids, amino acids and sugars (Fig. 3). The highest signal intensities for nucleotides and bases were observed when using the MeOH-H_2O method (Fig. 3). In light of the above results the MeOH-H_2O method was used to extract metabolites from pluripotent stem cell samples (Fig. 4).

2.2 Development of the Analysis Method

Quantitation of metabolites on the glycolysis, citric acid cycle and pentose phosphate pathway, which comprise central metabolism, is a very important part of intracellular metabolome analysis. It has been reported that metabolites involved in the above-mentioned pathways changed in undifferentiated and differentiated pluripotent stem cells[6), 9), 13), 14)]. Identification and quantitation of the above-mentioned metabolites from a cell extract sample requires standard MS spectra for the derivatized forms of those metabolites, as well as their retention indices, optimum quantitative ion and optimum confirmation ion. Shimadzu's GC/MS metabolite database is an assistive tool for metabolome analysis that includes all the information needed for the above-described qualitative and quantitative analyses. The original version of this GC/MS metabolite database mainly targets

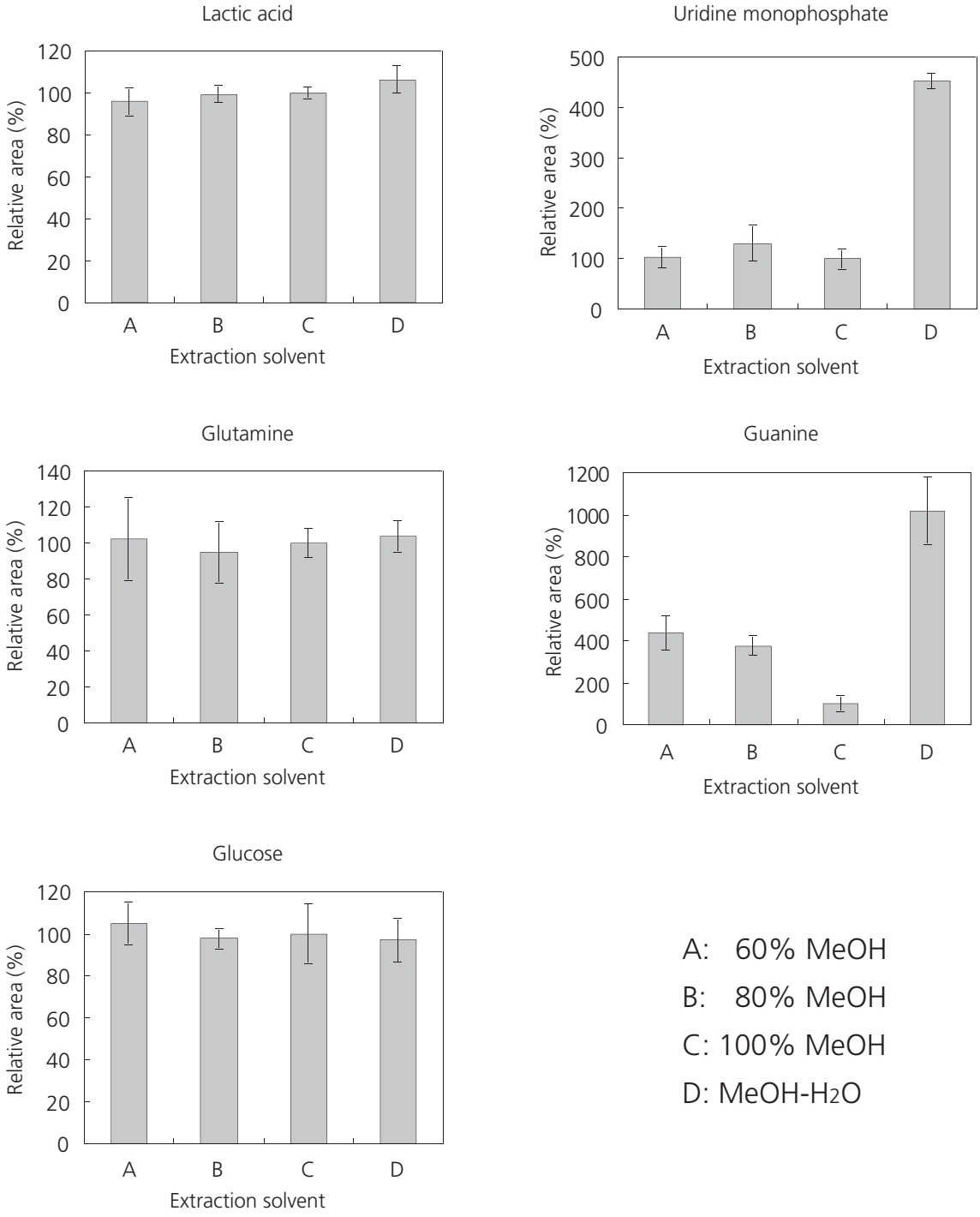


Fig. 3 Optimization of extraction steps for intracellular metabolites

urinary metabolites, so the above-mentioned metabolites involved in the glycolysis and pentose phosphate pathway are not included. We chose to develop an analysis method that focused on intracellular metabolites and used GC-MS to acquire the MS spectra of derivatized standard materials, determining parameters such as the retention time, retention index, quantitative ion and confirmation ion from the data acquired. This process was performed for each standard material and the findings combined to create a metabolome analysis method suitable for pluripotent stem cells. The analysis method we developed for intracellular metabolites developed in this study was combined with that for blood serum metabolites developed in joint research with Kobe University School of Medicine, and Shimadzu's original GC/MS metabolite database. We then commercialized this as the GC/MS metabolite database Ver. 2 in September 2013. The number of registered compounds in the database was increased from 250 to 511 (Fig. 5). One of the features of this database is that it contains metabolites detected in a wide range of biological samples such as biofluids and cultured cells, permitting the accurate identification of more metabolites in living organisms.

The GC/MS metabolite database Ver. 2 was used to analyze metabolites in human ES cell extracts. We cultivated human ES cell lines (KhES-1) with mTeSR1 on matrigel-coated 60 mm culture dishes

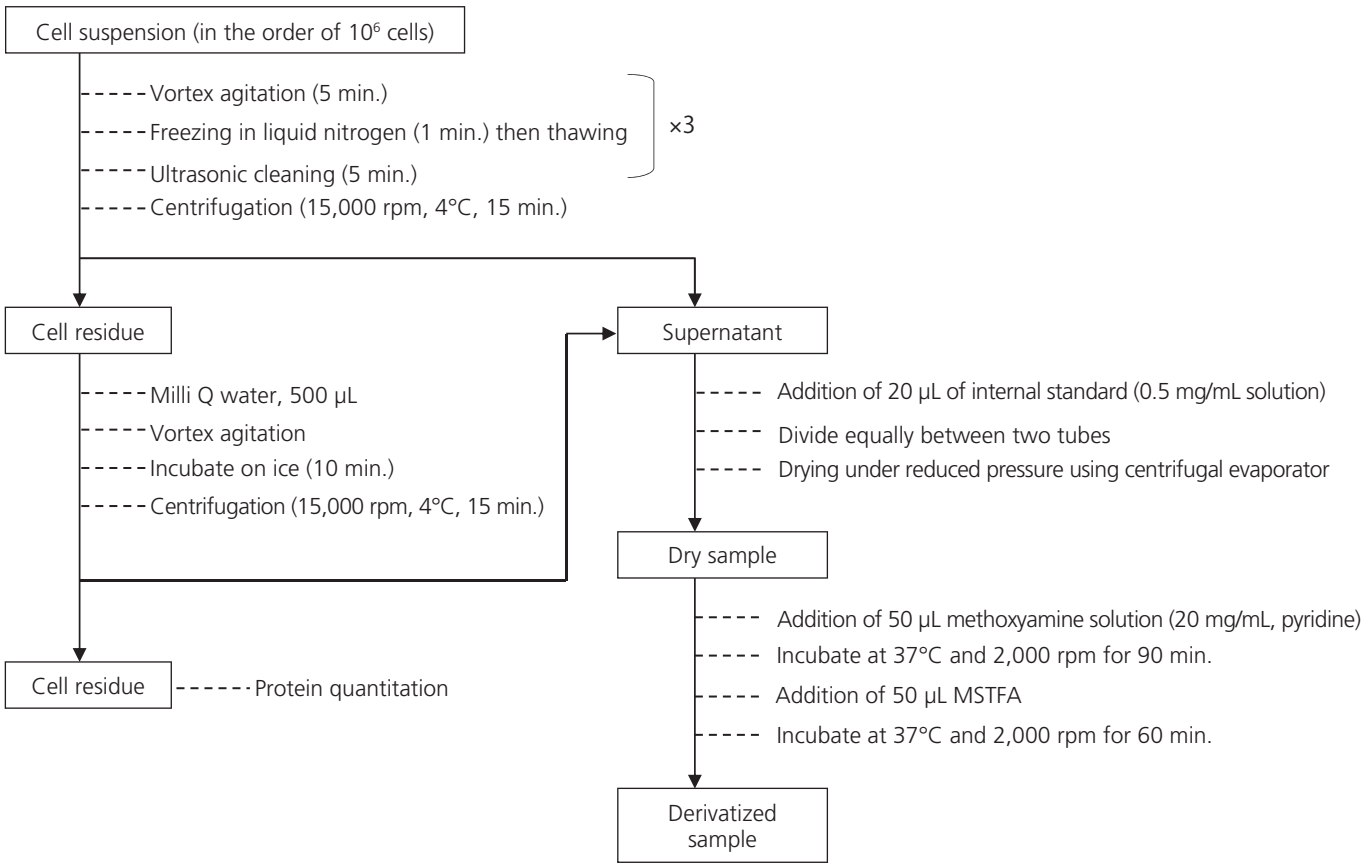
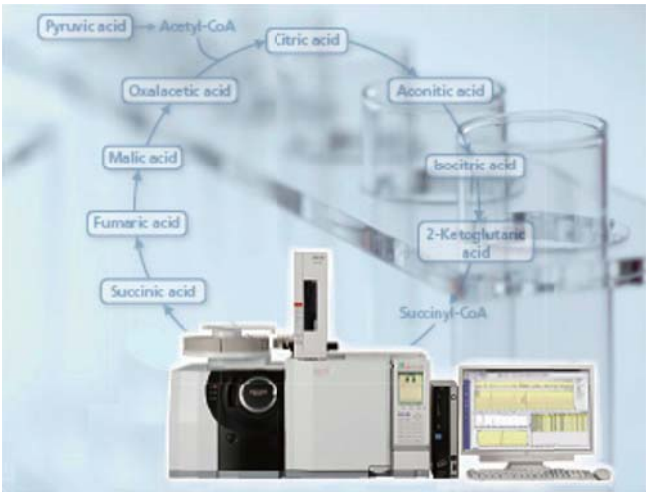


Fig. 4 Pre-treatment flow for metabolome analysis

and identified 104 metabolites in human ES cell extract, which included 2-Isopropylmalic acid added as an internal standard (Table 1). The metabolites we identified included compounds, amino acids, organic acids, sugars and fatty acids involved in the above-mentioned glycolysis, citric acid cycle and pentose phosphate pathway. We were able to evaluate a broad range of primary metabolites.

2.3 Reproducibility of Analysis Including the Pretreatment Process

To verify comprehensively the pretreatment method and analysis method developed in sections 2.1 and 2.2, we examined the reproducibility of the analysis system including the pretreatment process. Analysis was performed on individual samples prepared from four culture dishes using the methods described in this article. As shown in Fig. 6A, the TIC profile obtained from each sample matched well when overlaid. The mean standard deviation of the area for each compound was also approximately 10 % (Fig. 6B), which confirms the analysis system including pretreatment process was highly reproducible. The above results show we developed a method of metabolome analysis of pluripotent stem cells that is both comprehensive and reproducible.

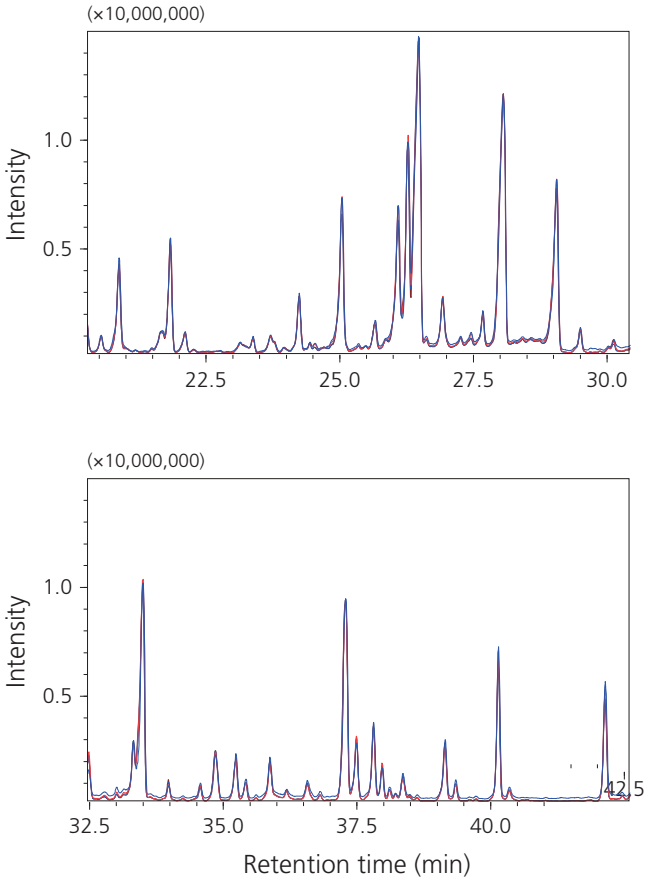


Registered Compound	Derivative	Measurement	Number Registered
Organic acids, fatty acids, amino acids, sugars, etc.	TMS	Scan	428
		MRM	193
Fatty acids	Methylation	Scan	50
		MRM	50
Amino acids	EZ:faast™	Scan	33

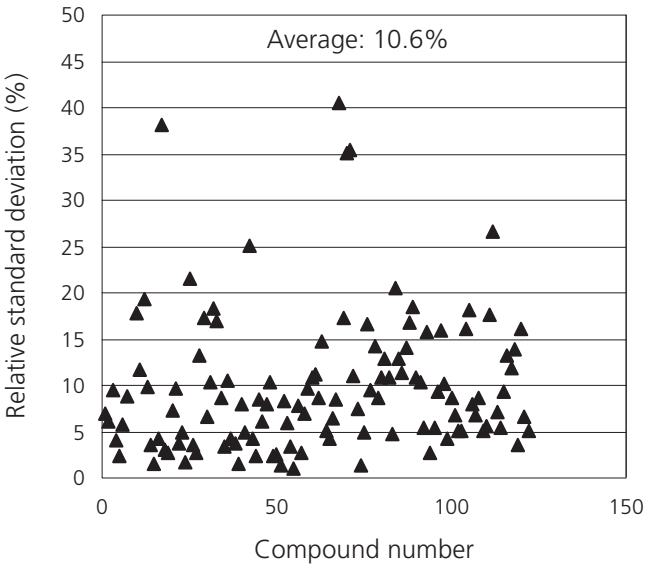
Fig. 5 GC/MS Metabolite Database Ver. 2

Table 1 List of metabolites identified from hESC extract

1	Adenine
2	Adenosine monophosphate
3	Adenosine
4	Alanine
5	2-Aminoadipic acid
6	4-Aminobutyric acid
7	3-Aminopropanoic acid
8	1,5-Anhydro-glucitol
9	Arachidonic acid
10	Arginine
11	Ascorbic acid
12	Asparagine
13	Aspartic acid
14	Cadaverine
15	Cholesterol
16	Citric acid
17	Cystathionine
18	Cysteine
19	Dihydroxyacetone phosphate
20	Elaidic acid
21	Fructose 1-phosphate
22	Fructose 6-phosphate
23	Fructose
24	Fumaric acid
25	Glucaric acid
26	Glucosamine
27	Glucose 6-phosphate
28	Glucose
29	Glucuronic acid lactone
30	Glucuronic acid
31	Glutamic acid
32	Glutamine
33	Glutaric acid
34	Glyceraldehyde
35	Glyceric acid
36	Glycerol 2-phosphate
37	Glycerol 3-phosphate
38	Glycine
39	Glycolic acid
40	Glycyl-Glycine
41	1-Hexadecanol
42	Histidine
43	Homocysteine
44	2-Hydroxyadipic acid
45	3-Hydroxybutyric acid
46	2-Hydroxyglutaric acid
47	3-Hydroxyisobutyric acid
48	3-Hydroxyisovaleric acid
49	3-Hydroxypropionic acid
50	Hypotaurine
51	Inositol
52	Isocitric acid
53	Isoleucine
54	Isomaltose
55	2-Isopropylmalic acid
56	2-Ketoglutaric acid
57	Kynurenic acid
58	Kynurenine
59	Lactic acid
60	Lauric acid
61	Leucine
62	Lysine
63	Maleic acid
64	Malic acid
65	Maltose
66	Margaric acid
67	Methionine
68	7-Methylguanine
69	3-Methyl-2-oxovaleric acid
70	Monostearin
71	Myristic acid
72	N-Acetylaspartic acid
73	Niacinamide
74	Nonanoic acid
75	Octadecanol
76	Octanoic acid
77	Oleic acid
78	O-Phosphoethanolamine
79	O-Phospho-Serine
80	Ornithine
81	Oxalic acid
82	5-Oxoproline
83	Palmitic acid
84	Palmitoleic acid
85	Pantothenic acid
86	Phenylalanine
87	Phosphoenolpyruvic acid
88	3-Phosphoglyceric acid
89	Phosphoric acid
90	Proline
91	Putrescine
92	Pyruvic acid
93	Ribose 5-phosphate
94	Ribose
95	Ribulose
96	Sedoheptulose 7-phosphate
97	Serine
98	Sorbitol
99	Stearic acid
100	Threonic acid
101	Threonine
102	Tyrosine
103	Valine
104	Xylitol



(a) Overlay of TICs



(b) Relative standard deviation for each compound

Fig. 6 Reproducibility of metabolomics data using the newly developed analysis method

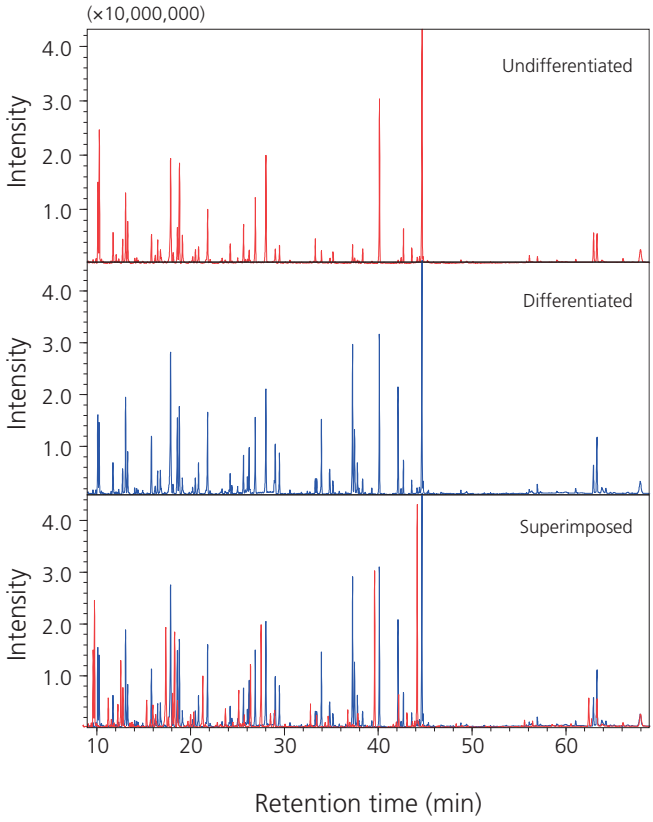
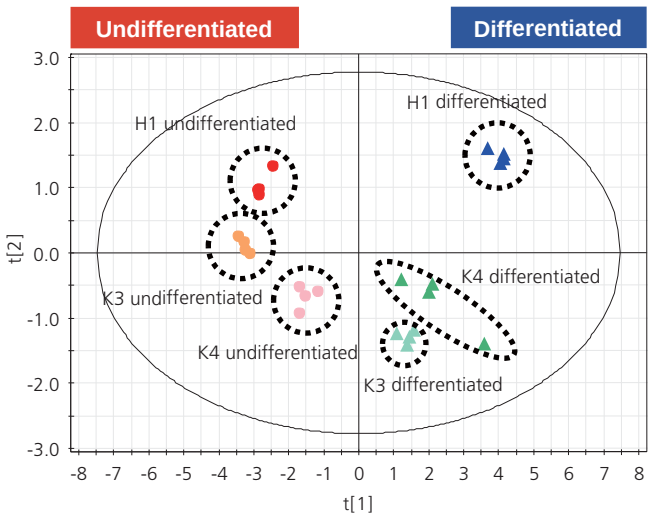


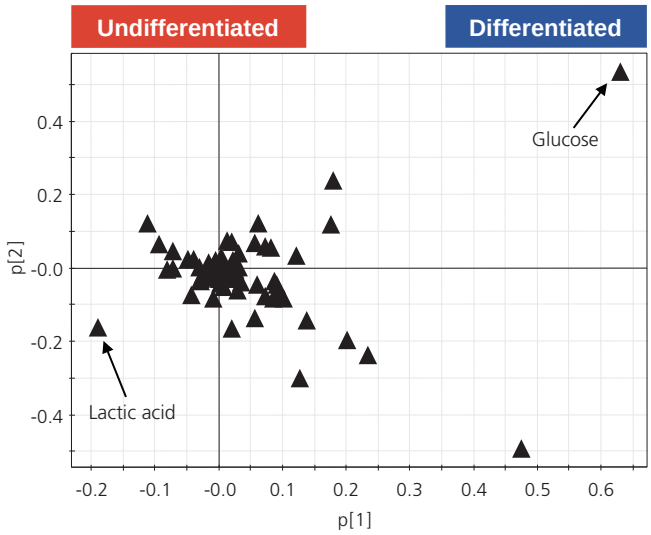
Fig. 7 TIC from the undifferentiated H1 hESCs and their differentiated counterparts

3. Comparative Metabolome Analysis of Undifferentiated and Differentiated Cells

To confirm the possibility of applying metabolome analysis for quality evaluation of human pluripotent stem cells, we examined whether the analysis method developed can be used to distinguish between undifferentiated and differentiated human ES cells. To eliminate the effect of medium components on metabolome analysis data as much as possible, cultivation was performed using the same base media for both undifferentiated and differentiated cells. Specifically, undifferentiated cells were cultured using ES cell medium[15] conditioned with mouse embyonic fibroblasts (MEF-conditioned medium), and differentiation was induced by the addition of retinoic acid in ES cell medium. As shown in Fig. 7, there were clear differences in TIC results obtained from undifferentiated H1 cells and their differentiated counterparts. Similar results were also obtained from KhES-3 and KhES-4 ES cells (results not shown). A principal component analysis of the metabolome analysis data distinguished clearly between undifferentiated and differentiated cells on the first principal component axis (Fig. 8A). Metabolites that contributed to this distinction were lactic acid (characteristic to undifferentiated state) and glucose (characteristic to differentiated state) (Fig. 8B). These results suggest that glucose, a starting material in glycolysis, is consumed quickly in undifferentiated cells and that pyruvic acid, an end material in glycolysis, is converted to lactic acid without entering the citric acid cycle. This interpretation conforms with the view that pluripotent stem cells rely on glycolysis[9), 13), 14)]. These results show that metabolome analysis technology is capable of evaluating between undifferentiated and differentiated human pluripotent stem cells.



(a) Score plot



(b) Loading plot

Fig. 8 Principal component analysis based on the metabolome analysis data of undifferentiated hESCs and their differentiated counterparts

4. Conclusion

This article describes a method of metabolome analysis suitable for pluripotent stem cells using GC-MS. We successfully developed an analysis system that is highly reproducible and compatible with a variety of metabolites, including metabolites involved in the principal metabolic pathways (glycolysis, citric acid cycle and pentose phosphate pathway). We showed the analysis method can distinguish between the undifferentiated state and differentiated state of several human ES cell lines, confirming the potential for applying metabolome analysis for quality evaluation of human pluripotent stem cells. However, obstacles remain that must be overcome before this analysis method is applied in the field of regenerative medicine.
Firstly, the cell recovery method must be improved. We used Teng's method[10] due to its convenience, but to acquire accurate metabolome information from cultured cells will require the process between medium removal and stopping intracellular metabolic activity to occur more rapidly. Not all metabolomics researchers who study cultured cells recognize the importance of the cell recovery process, as evidenced by trypsin detachment being the prevailing cell recovery method used in reported research[5), 6), 8)] on the metabolome analysis of pluripotent stem cells. A universal standard pretreatment method needs to be developed and implemented.
Secondly, though not mentioned in this article, the intracellular metabolome is easily affected by medium components. When searching for metabolome biomarkers specific to certain cell lineages, it would be unreasonable to cultivate all cell lines in the same medium composition. It is important that results are evaluated in terms of whether they vary due to the cell line or due to the effects of the medium composition used.
For pluripotent stem cell applications in regenerative medicine, a technology is needed that evaluates and removes a very small number of residual undifferentiated cells from amongst a large number of graft cells. This technology will necessitate the development of more sensitive biomarkers capable of distinguishing between differentiated and undifferentiated cells. Such technology is likely to require an innovation that allows us to acquire metabolome information on the level of individual cells, rather than the current method that calculates the mean number present among a given cell population.
We plan to work towards resolving the above issues and to further develop analyses that focus on the cell culture supernatant as well as the intracellular environment, with the objective of gathering more comprehensive metabolome information on pluripotent stem cells. Because the culture supernatant provides a convenient opportunity for analyzing change over time that does not involve cell destruction, we expect research in this area to provide information about cells that is different to the knowledge obtained in the present study.

The GC/MS metabolite database Ver. 2 includes results obtained from joint research with external research institutions, in addition to results obtained during this study. We wish to express our gratitude to all parties that contributed in the development of this product. This paper contains results obtained from the NEDO project "Fundamental Technology for Promoting Industrial Application of Human Stem Cells."

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