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Application News

No. C209

Liquid Chromatography Mass Spectrometry

Evaluation of Undifferentiated State of Human iPS Cells Using C2MAP™ Cell Culture Media Analysis Platform

Background

Development of technologies for the preparation and supply of iPS cells with high quality and a large amount is an essential requirement for commercialization of regenerative medicine. Conventionally, manual techniques such as Gene Expression analysis had been used to evaluate the status of cell differentiation, but because these are invasive techniques that cause cell disruption, they were generally applied as evaluation methods after culturing was completed. We developed the C2MAP cell culture media analysis platform using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with the aim of establishing a non-invasive cell status evaluation method based on component analysis of the cell culture supernatant.

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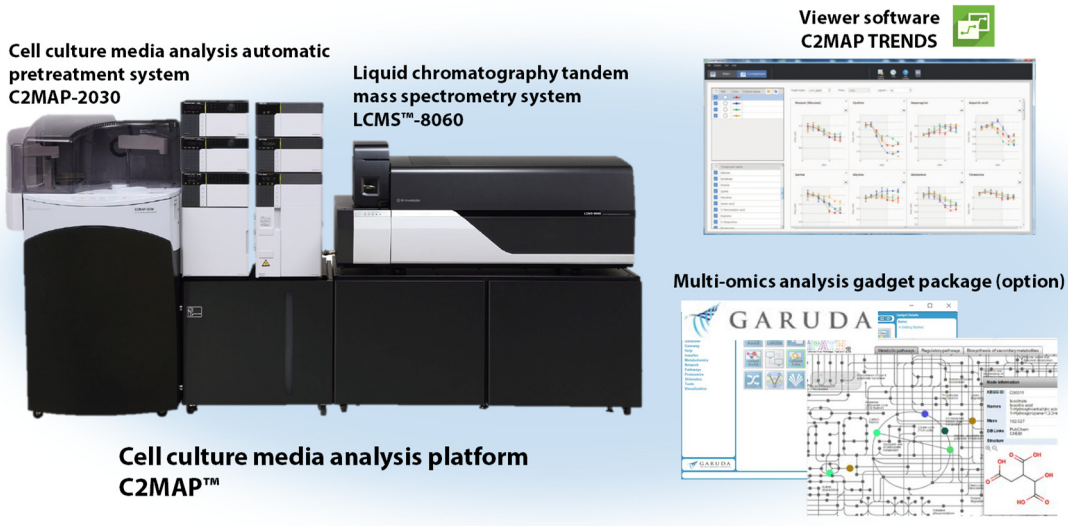
C2MAP Cell Culture Media Analysis Platform

With the C2MAP cell culture media analysis platform, simultaneous analysis of 95 components by LC-MS/MS can be conducted automatically from the sample preparation stage simply by setting the cell culture supernatant. Simple searches for candidate biomarkers are also possible by using the C2MAP TRENDS viewer software and Multi-omics Analysis Gadget Package statistical analysis tools. In this article, we studied the possibility of evaluating the undifferentiated state of human iPS cells by using C2MAP, C2MAP TRENDS, and the Multi-omics Analysis Gadget Package respectively.



Experimental Method

Using the cell culture medium TeSR™-E8™, human iPS cells were cultured for 6 days while maintaining the undifferentiated state. In order to search distinctive candidate biomarkers for the undifferentiated and differentiated states, a liquid factor such as a cytokine or a low molecular weight compound was added to the culture conditions shown in Table 1, and differentiation-inducing stimuli were applied to each germ layer system. The culture medium of each culture system was completely replaced every 24 hours, and the spent cell culture supernatant was collected for use as measurement samples.

The collected culture supernatant was supplied to C2MAP and was used in a simultaneous analysis of the 95 components listed in Table 2.

Following that analysis, data analyses were carried out using C2MAP TRENDS and the Multi-omics Analysis Gadget Package in order to search for components that showed significant differences between samples.

Table 1 Culture Conditions

Cell line	: Human iPS cell (PFX#9 line)
Medium	: TeSR™-E8™
Scaffold	: Vitronectin-N
Initial seeding	: 1×10^5 cells/well
Plate type	: 6 well plate

Supernatant samples and data were generously provided by Dr. Shin Kawamata of the Foundation for Biomedical Research and Innovation at Kobe.

Table 2 List of Registered Compounds

No.	Compound	Class	No.	Compound	Class	No.	Compound	Class
IS	2-Isopropylmalic acid	Internal standard	32	N-Acetylaspartic acid	Amino acid	64	Cytidine	Nucleic acid associated compound
1	Gluconic acid	Sugar	33	N-Acetylcysteine	Amino acid	65	Cytidine monophosphate	Nucleic acid associated compound
2	Glucosamine	Sugar	34	Ornithine	Amino acid	66	Deoxycytidine	Nucleic acid associated compound
3	Hexose (Glucose)	Sugar	35	Oxidized glutathione	Amino acid	67	Guanine	Nucleic acid associated compound
4	Sucrose	Sugar	36	Phenylalanine	Amino acid	68	Guanosine	Nucleic acid associated compound
5	Threonic acid	Sugar	37	Pipecolic acid	Amino acid	69	Guanosine monophosphate	Nucleic acid associated compound
6	2-Aminoadipic acid	Amino acid	38	Proline	Amino acid	70	Hypoxanthine	Nucleic acid associated compound
7	4-Aminobutyric acid	Amino acid	39	Serine	Amino acid	71	Inosine	Nucleic acid associated compound
8	4-Hydroxyproline	Amino acid	40	Threonine	Amino acid	72	Thymidine	Nucleic acid associated compound
9	5-Glutamylcysteine	Amino acid	41	Tryptophan	Amino acid	73	Thymine	Nucleic acid associated compound
10	5-Oxoproline	Amino acid	42	Tyrosine	Amino acid	74	Uracil	Nucleic acid associated compound
11	Alanine	Amino acid	43	Valine	Amino acid	75	Uric acid	Nucleic acid associated compound
12	Alanyl-glutamine	Amino acid	44	4-Aminobenzoic acid	Vitamin	76	Uridine	Nucleic acid associated compound
13	Arginine	Amino acid	45	Ascorbic acid	Vitamin	77	Xanthine	Nucleic acid associated compound
14	Asparagine	Amino acid	46	Ascorbic acid 2-phosphate	Vitamin	78	Xanthosine	Nucleic acid associated compound
15	Aspartic acid	Amino acid	47	Biotin	Vitamin	79	Penicillin G	Antibiotic
16	Citrulline	Amino acid	48	Choline	Vitamin	80	2-Aminoethanol	Other
17	Cystathionine	Amino acid	49	Cyanocobalamin	Vitamin	81	2-Ketoisovaleric acid	Other
18	Cysteine	Amino acid	50	Ergocalciferol	Vitamin	82	3-Methyl-2-oxovaleric acid	Other
19	Cystine	Amino acid	51	Folic acid	Vitamin	83	4-Hydroxyphenyllactic acid	Other
20	Glutamic acid	Amino acid	52	Folinic acid	Vitamin	84	Citric acid	Other
21	Glutamine	Amino acid	53	Lipoic acid	Vitamin	85	Ethylenediamine	Other
22	Glutathione	Amino acid	54	Niacinamide	Vitamin	86	Fumaric acid	Other
23	Glycine	Amino acid	55	Nicotinic acid	Vitamin	87	Glyceric acid	Other
24	Glycyl-glutamine	Amino acid	56	Pantothenic acid	Vitamin	88	Histamine	Other
25	Histidine	Amino acid	57	Pyridoxal	Vitamin	89	Isocitric acid	Other
26	Isoleucine	Amino acid	58	Pyridoxine	Vitamin	90	Lactic acid	Other
27	Kynurenine	Amino acid	59	Riboflavin	Vitamin	91	Malic acid	Other
28	Leucine	Amino acid	60	Tocopherol acetate	Vitamin	92	O-Phosphoethanolamine	Other
29	Lysine	Amino acid	61	Adenine	Nucleic acid associated compound	93	Putrescine	Other
30	Methionine	Amino acid	62	Adenosine	Nucleic acid associated compound	94	Pyruvic acid	Other
31	Methionine sulfoxide	Amino acid	63	Adenosine monophosphate	Nucleic acid associated compound	95	Succinic acid	Other

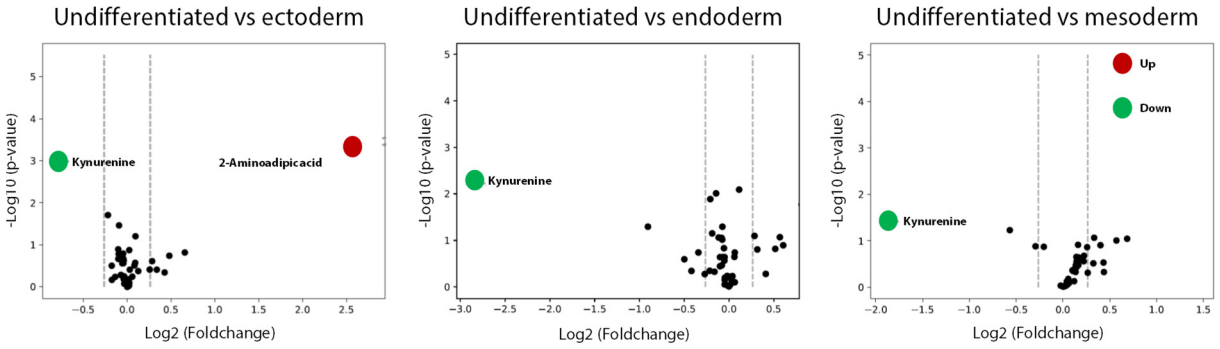


Fig. 1 Volcano Plots

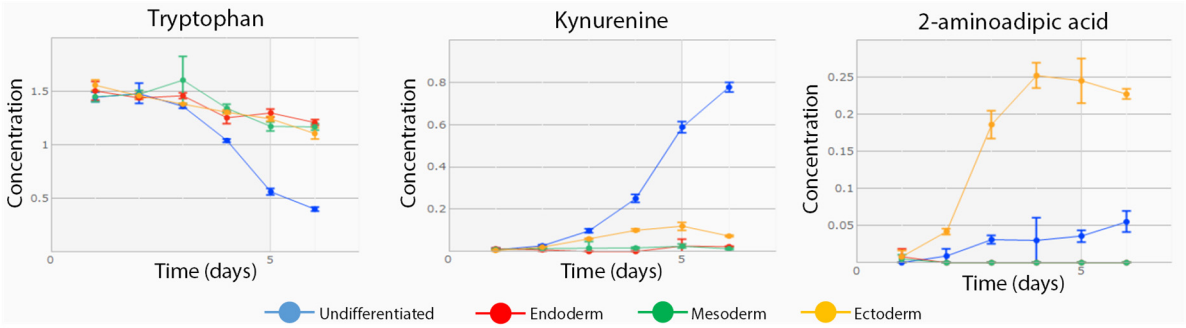


Fig. 2 Time Course of Candidate Biomarkers with During Cell Culture

Results

Search for Candidate Biomarkers in Culture Supernatant

It was possible to detect 55 of the 95 components in the simultaneous multi-component analysis of the culture supernatant by C2MAP. In order to search for biomarkers that enable early judgment of the cell differentiation status after the start of culturing, the area ratios of each component after culturing for 3 days were used in an analysis by the Volcano Plot tool, and an intergroup comparison of two groups of undifferentiated iPS cells and iPS cells in which differentiation was induced in each germ layer system was conducted (Fig. 1). As a result, kynurenine was specifically strongly detected in the supernatant of the undifferentiated iPS cells, and 2-aminoadipic acid was specifically strongly detected in the supernatant of the ectodermal differentiated cells.

Table 3 Concentrations at Day 4

Compound	Concentration (external standard method)	Concentration (standard addition method)
Kynurenine	0.22 μM	0.23 μM
2-Aminoadipic acid	0.25 μM	0.19 μM
Tryptophan	0.90 μM	1.05 μM

* Kynurenine and tryptophan are the measured values for culture samples maintained in the undifferentiated state, and 2-aminoadipic acid is the measured values for ectodermal differentiated samples.

The components for which intergroup differences were confirmed, including the above-mentioned compounds, were quantified by the external standard method, and their time-dependent changes with the during cell culture were plotted (Fig. 2). In the supernatant of the undifferentiated iPS cells, the results showed that tryptophan was consumed as the culturing process proceeded, and secretion of kynurenine, the metabolite of tryptophan, increased. In the supernatant of the ectodermal differentiated cells, the results showed that the concentration of 2-aminoadipic acid increased as culturing progressed. Because the pattern of time-dependent changes was the same as in Fig. 2, including the data obtained by the standard addition method, these results demonstrated that the obtained graphs show the actual change in concentrations of the supernatants (Table 3).

Role of Kynurenine in Undifferentiated iPS Cells

In order to investigate the role of kynurenine, which was identified as a candidate biomarker for the undifferentiated state, we focused on the metabolic system of kynurenine. Suppression of cell growth was confirmed when culturing was conducted while maintaining the undifferentiated state by adding the inhibitor IDO1, which is a metabolizing enzyme for kynurenine from tryptophan, to the culture medium (Fig. 3). Moreover, an analysis of the supernatant confirmed that secretion of kynurenine is suppressed by addition of the IDO1 inhibitor (Fig. 4). Therefore, it was suggested that kynurenine plays an essential role in the reproduction of undifferentiated iPS cells.

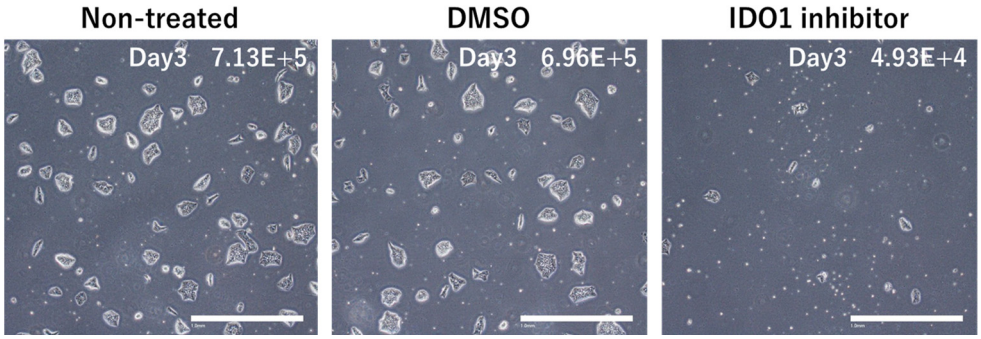


Fig. 3 Microscopic Images

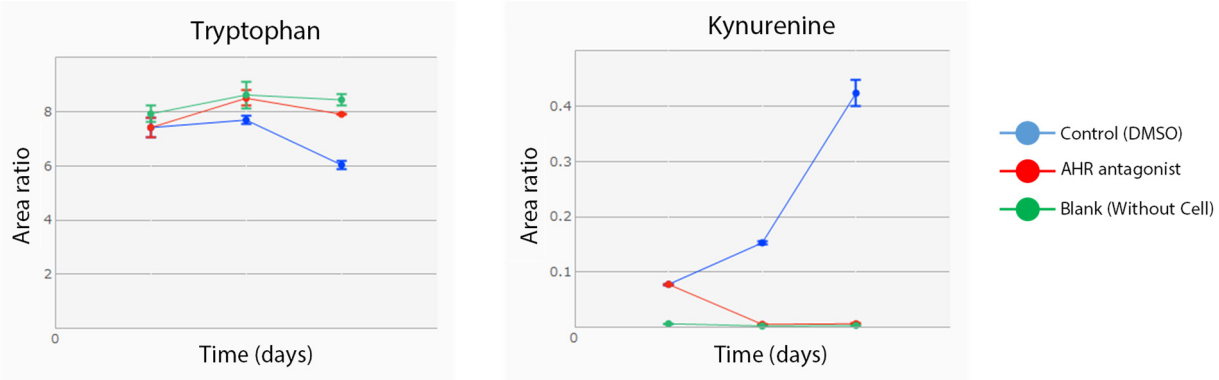


Fig. 4 Time Course of Tryptophan and Kynurenine with During Cell Culture

It is known that kynurenine forms the Kyn-AhR complex by bonding with the Aryl-hydrocarbon Receptor (AhR), and thereby increases some gene expression after transported into the nucleus. To investigate the function of the Kyn-AhR complex in undifferentiated iPS cells, we conducted culturing while maintaining the undifferentiated state by adding the AhR specific antagonist to the culture medium. As a result, we confirmed that addition of the AhR antagonist suppresses cell growth (Fig. 5), and secretion of kynurenine is also suppressed (Fig. 6). In addition, an analysis by the chromatin immunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR) method confirmed that the Kyn-AhR complex increases expression of transcription factors (*POU5F1*, *NANOG*, *EP300*) which are related to maintenance of the undifferentiated state and self-replication and *AHR* and *IDO1*.

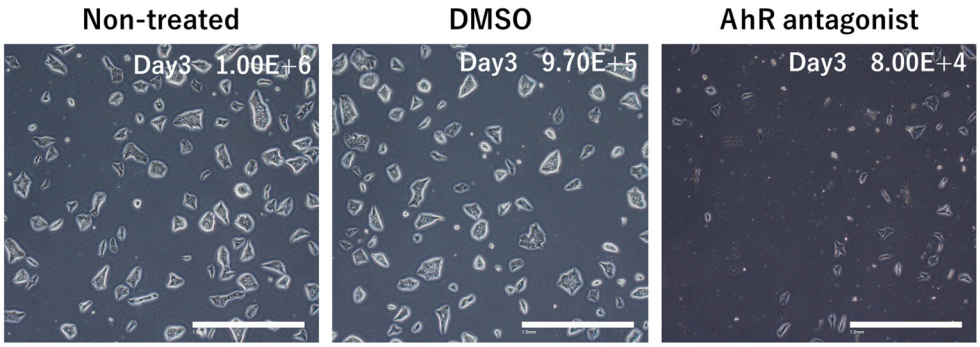


Fig. 5 Microscopic Images

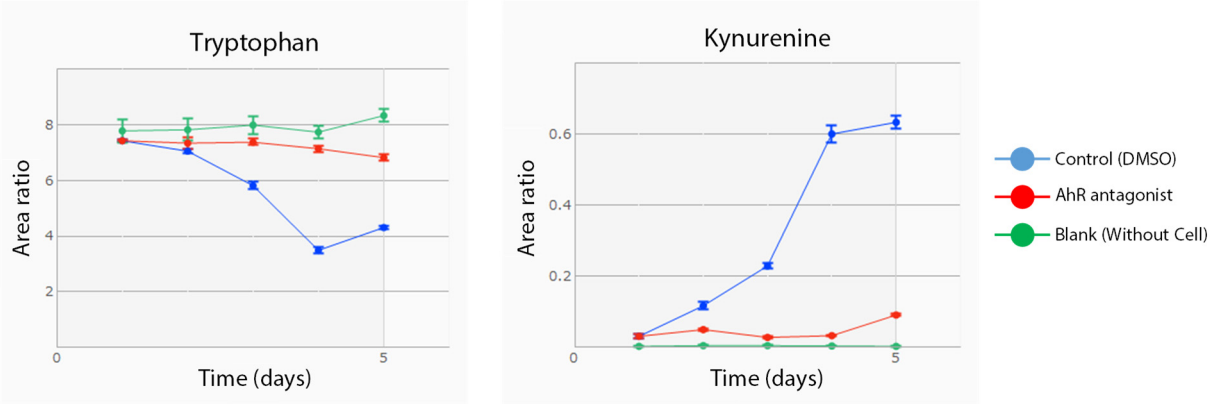


Fig. 6 Time Course of Tryptophan and Kynurenine with During Cell Culture

Metabolic System of 2-Aminoadipic Acid in Ectodermal Differentiated Cells

We also investigated the role of 2-aminoadipic acid, which was identified as a candidate biomarker for ectodermal differentiation, with a particular focus on its metabolic system. 2-aminoadipic acid is thought to have two formation paths, namely, metabolism of lysine and metabolism of kynurenine. Because no difference in the time course of lysine consumption was observed in a comparison of undifferentiated iPS cells and ectodermal differentiated cells (Fig. 7), we inferred that 2-aminoadipic acid is formed mainly from the kynurenine metabolic pathway.

Ectodermal differentiation-inducing stimulus was applied by adding an inhibitor for KAT2, which is one of the above-mentioned metabolizing enzyme groups, to the medium. As a result, suppression of the secretion of 2-aminoadipic acid by addition of the KAT2 inhibitor (Fig. 7) and a certain degree of suppression of differentiation to ectoderms was observed (Fig. 8). Accordingly, it was suggested that the decomposition pathway of kynurenine, which plays a key role in maintaining the undifferentiated state, is activated by the differentiation-inducing stimulus, and 2-aminoadipic acid is formed accompanying the decomposition of kynurenine.

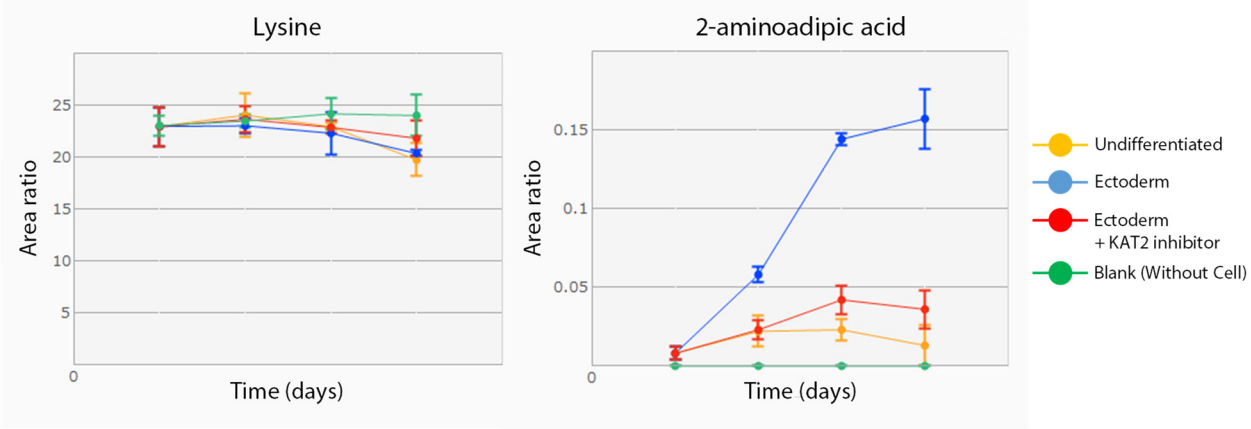


Fig. 7 Time Course of Lysine and 2-Aminoadipic Acid with During Cell Culture

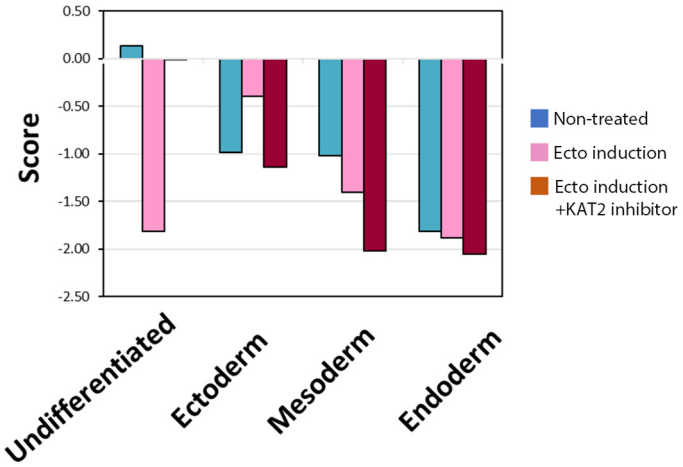


Fig. 8 Results of Gene Expression Profiling

Action Mechanism of Undifferentiation Maintenance and Start of Differentiation Induction

Synthesizing the results obtained up to this point, the kynurenine which is formed from tryptophan in undifferentiated iPS cells forms the Kyn-AhR complex by bonding with AhR in the cytoplasm. The Kyn-AhR complex migrates into the nucleus and increases the amount of expression of transcription factors related to maintenance of the undifferentiated state and self-replication. At the same time, the Kyn-AhR complex also increases expression of *AhR* and *IDO1*, which maintain the undifferentiated state by further strengthening the above-mentioned pathway. Furthermore, surplus kynurenine is secreted in the culture solution and activates the undifferentiation maintenance loop via kynurenine in a sustained manner. On the other hand, when an ectodermal differentiation-inducing stimulus is applied, this increases the expression of enzyme groups related to kynurenine metabolism, including KAT2, and the kynurenine in cells is metabolized and 2-aminoadipic acid forms. Due to the decrease in the concentration of kynurenine, the undifferentiation maintenance loop via kynurenine ceases to function, and differentiation from the undifferentiated state to ectoderms is induced in the cells (Fig. 9).

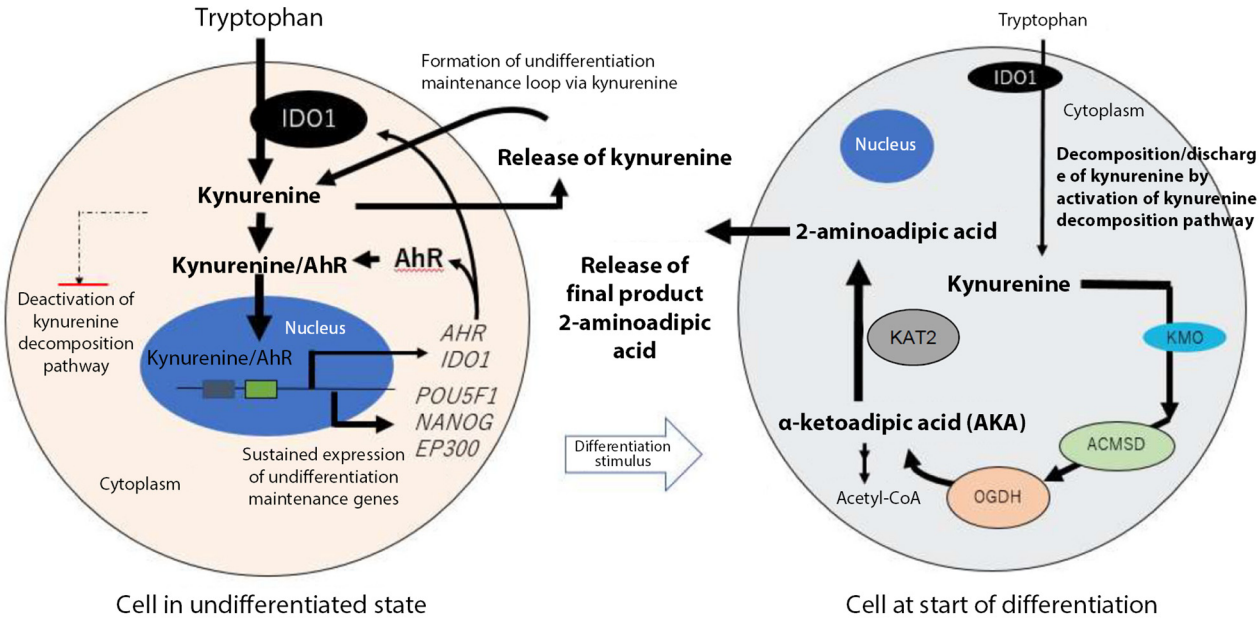


Fig. 9 Correlation of Gene Expression and Metabolic Pathways of Kynurenine and 2-Aminoadipic Acid

Conclusion

A simultaneous multi-component analysis of cell culture supernatants was conducted using C2MAP, and as a result, kynurenine and 2-aminoadipic acid were identified as biomarkers that indicate distinctive changes in undifferentiated iPS cells and ectodermal differentiated cells, respectively. This study showed that the identified biomarkers are factors which are closely related to the undifferentiated and ectodermal differentiated states of cells. Thus, these results demonstrated that non-invasive evaluation of the states of cells is possible by using the C2MAP system.

<Reference>

1) T. Yamamoto, K. Hatabayashi, M. Arita, N. Yajima, C. Takenaka, T. Suzuki, M. Takahashi, Y. Oshima, K. Hara, K. Kagawa, S. Kawamata : Kynurenine signaling through the aryl hydrocarbon receptor maintains the undifferentiated state of human embryonic stem cells, Sci. Signal. 12, eaaw3306 (2019).

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