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An in vitro *skin* sensitization assay termed EpiSensA for broad sets of chemicals including lipophilic chemicals and pre/pro-haptens

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ABSTRACT

To evaluate chemicals (e.g. lipophilic chemicals, pre/pro-haptens) that are difficult to correctly evaluate using in vitro skin sensitization tests (e.g. DPRA, KeratinoSens or h-CLAT), we developed a novel in vitro test termed "Epidermal Sensitization Assay: EpiSensA" that uses reconstructed human epidermis. This assay is based on the induction of multiple marker genes (ATF3, IL-8, DNAJB4 and GCLM) related to two keratinocyte responses (inflammatory or cytoprotective) in the induction of skin sensitization. Here, we first confirmed the mechanistic relevance of these marker genes by focusing on key molecules that regulate keratinocyte responses in vivo ($P2X_7$ for inflammatory and Nrf2 for cytoprotective responses). The up-regulation of ATF3 and IL-8, or DNAJB4 and GCLM induced by the representative sensitizer 2,4-dinitrochlorobenzene in human keratinocytes was significantly suppressed by a $P2X_7$ specific antagonist KN-62, or by Nrf2 siRNA, respectively, which supported mechanistic relevance of marker genes. Moreover, the EpiSensA had sensitivity, specificity and accuracy of 93%, 100% and 93% for 29 lipophilic chemicals (logKow ≥ 3.5), and of 96%, 75% and 88% for 43 hydrophilic chemicals including 11 pre/pro-haptens, compared with the LLNA. These results suggested that the EpiSensA could be a mechanism-based test applicable to broad sets of chemicals including lipophilic chemicals and pre/pro-haptens.

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1. Introduction

Allergic contact dermatitis (ACD) resulting from skin sensitization is a common occupational and environmental health problem (Peiser et al., 2010). To date, the evaluation of skin sensitizers has been mainly dependent on animal tests such as the local lymph node assay (LLNA) (Kimber et al., 2002). However, the ethical issues involved, and the European Union ban on animal testing for cosmetic ingredients (Cosmetics Regulation EC 1223/2009) have accelerated the development of non-animal tests for evaluating the skin sensitization potential of chemicals.

ACD is an adaptive immune response caused by skin sensitizers (haptens). Skin sensitizers that penetrate through the skin form hapten–protein complexes (haptenated proteins) and are captured by skin-resident dendritic cells (DCs) (Sasaki and Aiba, 2007). These DCs mature and migrate to skin draining lymph nodes, where the DCs present the peptides from haptenated proteins to naive T cells. Finally, antigen-specific T cells proliferate and disseminate into the peripheral circulation (Kimber et al., 2011). Repeated exposure to the same skin sensitizers after induction of sensitization can lead to the elicitation and symptoms of ACD (Toebak et al., 2009).

To date, a number of in vitro tests that focus on key events in the skin sensitization Adverse Outcome Pathway (AOP) (OECD, 2012a) have been developed. Among these tests, the Direct Peptide Reactivity assay (DPRA), which focuses on the first key event (molecular interaction with skin proteins), and the KeratinoSens™ assay, which is based on the second key event (keratinocyte response), were adopted as OECD Test Guidelines in February 2015 (OECD, 2015a, 2015b). Additionally, the human-Cell Line Activation Test (h-CLAT), which focuses on the third key event (dendritic cell activation), was approved by the OECD Working Group of the National Coordinators of the Test Guidelines Programme in April 2016 (ECVAM, 2016). It has been reported that these three tests show approximately 80% accuracy compared to the LLNA for over 140 chemicals (Takenouchi et al., 2015; OECD,

Abbreviations: ACD, allergic contact dermatitis; AOO, acetone:olive oil = 4:1; AOP, Adverse Outcome Pathway; ARE, antioxidant response element; ATF3, activating transcription factor 3; BKC, benzalkonium chloride; DCs, dendritic cells; DMSO, dimethyl sulfoxide; DNAJB4, dnaJ (Hsp40) homolog, subfamily B, member 4; DNCB, 2,4-dinitrochlorobenzene; DPRA, Direct Peptide Reactivity assay; DW, distilled water; ECVAM, European Centre for the Validation of Alternative Methods; EHA, 2-ethylhexyl acrylate; EpiSensA, Epidermal Sensitization Assay; GCLM, glutamate-cysteine ligase, modifier subunit; h-CLAT, human-Cell Line Activation Test; IL, interleukin; IM, isopropyl myristate; KCs, keratinocytes; LA, lactic acid; LLNA, local lymph node assay; MBT, 2-mercptbenzothiazole; MS, methyl salicylate; MTT, methylthiazolydiphenyl-tetrazolium; 4-NBB, 4-nitrobenzyl bromide; NHEK, normal human epidermal keratinocyte; Nrf2, nuclear factor E2-related factor 2; OA, octanoic acid; OXA, oxazolone; PPD, *p*-phenylenediamine; RhE, reconstructed human epidermis; SA, squalic acid; SLS, sodium lauryl sulfate.

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Table 1
Summary of the 72 tested chemicals.

Name	Cas no.	LLNA EC3 (%)	Log Kow[a]	EpiSensA												In vitro tests[e]		
				Vehicles	IC20 (%)[b]	Dose range (%)	I_{max}[c]				EC values (%)				Prediction[d]	DPRA	KeratinoSens™	h-CLAT
							ATF3	DNAJB4	GCLM	IL-8	ATF3 EC15	DNAJB4 EC2	GCLM EC2	IL-8 EC4				
Oxazolone[f]	15,646-46-5	0.003	1.51	AOO	0.18	0.024–0.78	7.4	1.2	1.4	**4.9**				0.006	P	P1	P2	P1
Tetrachlorosalicylanilide	1154-59-2	0.04	**5.87**	AOO	1.66	0.20–3.13	**397.3**	**3.2**	0.7	**28.2**	0.027	0.24		0.035	P	P1	P2	P1
4-Nitrobenzylbromide[f]	100-11-8	0.05	2.7	AOO	0.97	0.012–0.20	**64.6**	**14.7**	**15.2**	**6.9**	0.05	0.017	0.011	0.034	P	P1	P2	P1
2,4-Dinitrochlorobenzene[f]	97-00-7	0.05	2.27	AOO	0.083	0.012–0.20	**43.8**	**4.9**	**8.7**	**7.0**	0.026	0.016	0.012	0.002	P	P1	P2	P1
1,4-Dihydroquinone[g]	123-31-9	0.11	1.03	50%EtOH	0.15	0.02–0.20	0.5	**2.1**	**8.2**	1.0		0.06	0.01		P	P1	P2	P1
p-Phenylenediamine[f,g]	106-50-3	0.16	−0.39	AOO	0.81	0.10–1.56	2.5	**4.5**	**7.1**	3.5		0.035	0.046		P	P1	P2	P1
Hexyl salicylate	6259-76-3	0.18	**5.06**	AOO	NT	12.5–100	5.8	**2.3**	1.7	1.8		1.24			P	N1	N3	P1
Benzoyl peroxide	94-36-0	0.22	3.43	AOO	>5.0	0.63–5	6.6	1.1	1.4	**4.2**				4.72	P	P1	N2	N1
Lauryl gallate[g]	1166-52-5	0.3	**6.21**	AOO	>6.25	0.20–6.25	4.7	**4.4**	1.2	**11.9**		0.5		0.88	P	P1	P2	P1
Cobalt chloride	7646-79-9	0.6	0.85	DW	>25	1.56–25	**131.0**	**2.5**	1.7	**10.3**	6.4	10.66		7.49	P	P1	P3	P1
4-(Methylamino)phenol sulfate[g]	55-55-0	0.78	2.34	50%EtOH	0.62	0.09–0.78	**510.6**	**57.0**	**33.8**	**13.2**	0.2	0.06	0.059	0.27	P	P2	P2	N.D.
Methyldibromoglutaronitrile[f]	35,691-65-7	0.9	1.63	AOO	0.25	0.02–0.32	**53.9**	**7.0**	**11.5**	**7.1**	0.18	0.12	0.04	0.2	P	P1	P2	P1
Isoeugenol[f,g]	97-54-1	1.2	2.65	AOO	0.44	0.39–6.25	6.3	**4.0**	**6.3**	2.3		0.21	0.09		P	P1	P2	N1
Glyoxal[f]	107-22-2	1.4	−1.66	DW	4.44#	0.63–10	**34.6**	**2.4**	**8.0**	**8.3**	0.51	2.06	0.1	1.49	P	P1	P2	P1
Bisphenol A-diglycidyl ether	1675-54-3	1.5	**3.84**	AOO	>50	6.25–50	**40.4**	**10.6**	**3.5**	**58.0**	2.39	0.37	0.48	0.66	P	P1	P2	P1
2-Mercaptobenxothiazole[f]	149-30-4	1.7	2.86	AOO	0.41	0.16–0.63	6.1	**3.0**	1.5	**16.2**		0.04		0.15	P	P1	P2	P1
Ethylene diamine[g]	107-15-3	2.2	−1.62	DW	18.1	0.78–25	**370.5**	**3.1**	1.0	**74.8**	0.84	3.53		0.82	P	N1	P2	P1
Benzyl salicylate	118-58-1	2.9	**4.31**	AOO	NT	25–100	**19.3**	1.0	1.4	**88.0**	42.1			15.01	P	N1	P3	N1
Cinnamic aldehyde[f]	104-55-2	3	1.82	AOO	1.2	0.05–1.56	**77.0**	**10.3**	**8.6**	**6.8**	0.12	0.1	0.039	0.13	P	P1	P2	P1
3-Propylidenephthalide	17,369-59-4	3.7	2.03	AOO	3.21	0.39–6.25	**33.4**	**2.3**	1.8	**4.1**	0.99	0.98		1.5	P	N1	N2	P1
Farnesol[g]	4602-84-0	4.1	5.77	AOO	3.71	0.39–3.13	**129.2**	**2.2**	0.8	**147.1**	0.74	1.42		0.44	P	N3	P3	P3
Squaric acid	2892-51-5	4.3	−0.44	50%EtOH	>3.13	0.78–3.13	5.7	1.8	2.0	1.7					N	P2	N2	N4
Clotrimazole	23,593-75-1	4.8	**6.26**	AOO	>3.13	0.20–3.13	**101.1**	**17.7**	1.8	**10.7**	0.61	0.46		1.88	P	P3	N4	N3
Tetramethylthiuram disulfide[f]	137-26-8	5.2	1.7	AOO	0.9	0.05–1.56	**31.9**	**3.1**	**5.8**	**5.0**	0.18	0.23	0.07	0.64	P	P1	P2	P1
Resorcinol[g]	108-46-3	5.5	1.03	AOO	1.61	0.39–3.13	**26.1**	**2.1**	1.8	7.7	2.33	3.02		2.23	P	N2	N2	P1
Diethylenetriamine[g]	111-40-0	5.8	−2.13	DW	7.38	0.78–12.5	**92.7**	1.4	1.5	2.4	3.41				P	N2	N3	N1
Damascone	23,726-91-2	6.7	**4.42**	AOO	0.93	0.20–3.13	**205.7**	**202.7**	**49.5**	**19.9**	0.34	0.16	0.03	0.24	P	N.D.	P3	P3
Undec-10-enal	112-45-8	6.8	**4.12**	AOO	0.79	0.10–3.13	**133.2**	**3.8**	**3.0**	**115.7**	0.43	0.19	0.23	0.32	P	N1	P3	N1
12-Bromo-1-dodecanone	3344-77-2	6.9	**5.11**	AOO	7.9	6.25–50	**84.8**	**5.5**	**3.5**	**10.8**	6.18	2.07	2.37	4.64	P	P1	N.D.	N1
Tocopherol	10,191-41-0	7.4	**12.2**	AOO	>50	12.5–50	1.3	0.7	0.9	1.3					N	N1	N.D.	N1
1-Bromohexane	111-25-1	10	**3.63**	AOO	11.5	6.25–50	**22.4**	**3.0**	**6.4**	3.9	7.93	7.08	3.33		P	N1	P2	N1
Ethylhexylacylate	103-11-7	10	**4.09**	AOO	14.9	1.57–50	**174.4**	**83.9**	**28.9**	**25.3**	4.87	1.24	1.01	3.64	P	P1	P2	P1
Hexyl cinnamic aldehyde	101-86-0	11	**4.82**	AOO	NT	6.25–100	**20.1**	1.9	1.5	**9.6**	71.08	6.35		17.17	P	N1	P2	N1
Citral	5392-40-5	13	3.45	AOO	0.29	0.1–0.39	3.5	**3.3**	**8.2**	3.9		0.12	0.1		P	P1	P2	P1
Eugenol[f,g]	97-53-0	13	2.73	AOO	0.7	0.20–6.25	9.8	**2.1**	**2.1**	2.0		0.36	0.35		P	P1	N2	P1

Abietic acid	514-10-3	15	**6.46**	AOO	9.3	0.39–12.5	**106.4**	**2.8**	**5.8**	**71.2**	0.41	0.68	0.35	0.34	P	P1	P2	N1
Benzyl benzoate	120-51-4	17	**3.54**	AOO	NT	6.25–100	**25.2**	1.3	1.4	**32.0**	19.6			14.12	P	N1	P2	N1
Benzyl cinnamate	103-41-3	18	**4.06**	AOO	>50	3.1–50	8.8	1.2	1.7	**29.2**				6.86	P	N1	P3	N1
Lillial	80-54-6	19	**4.36**	AOO	1.94	0.39–3.13	5.6	1.3	0.9	**19.6**				0.88	P	N1	N2	P1
Dibutyl aniline	613-29-6	20	**5.12**	AOO	93.4	25–100	**111.3**	1.4	1.96	**105.6**	53.45			23.59	P	N1	N3	N1
Pentachlorophenol	87-86-5	20	**4.74**	AOO	0.42	0.10–0.78	**704.8**	5.0	0.8	**77.0**	0.048	0.11		0.02	P	P2	N2	P3
Phenyl benzoate	93-99-2	20	3.04	AOO	>25	1.56–25	**25.8**	4.0	**2.2**	**6.0**	5.16	1.48	2.56	5.34	P	P1	N3	P1
Cinnamic alcohol[f,g]	104-54-1	21	1.84	AOO	0.59	0.39–1.56	7.9	1.7	**9.7**	**4.5**			0.22	0.35	P	N1	P2	P1
Cyclamen aldehyde	103-95-7	22	**3.91**	AOO	3.47	0.39–3.13	**22.4**	2.6	1.9	**6.4**	0.61	0.18		0.51	P	P1	P2	N1
Imidazolidinyl urea	39,236-46-9	24	−8.28	DW	>25	6.25–25	**37.0**	1.7	1.1	**12.9**	8.06			7.04	P	P1	P2	P1
Undecylenic acid	112-38-9	25	**4.37**	AOO	0.16	0.05–0.39	**122.2**	2.9	**2.7**	**30.7**	0.2	0.27	0.27	0.2	P	N3	P3	N.D.
Ethyleneglycol dimethacrylate	97-90-5	28	2.21	AOO	3.27	0.20–6.25	**42.6**	10.4	**16.9**	2.0	2.05	0.11	0.19		P	P	P2	P1
Penicillin G	61-33-6	30	1.85	DW	>50	6.25–50	**91.1**	1.7	1.1	**7.6**	8.2			12.18	P	P2	N2	P3
Butyl glycidyl ether	2426-08-6	31	1.08	AOO	2.05	0.39–3.13	**504.8**	63.6	**18**	**21.5**	0.39	0.35	0.2	0.42	P	P1	P2	N1
Citronellole	106-22-9	43.5	**3.56**	AOO	0.6	0.20–1.56	5.8	2.5	1.95	2.3		0.19			P	N1	N3	P1
Isopropyl myristate	110-27-0	44	**7.17**	AOO	NT	25–100	1.7	1.9	1.8	1.1					N	N2	N2	N.D.
Bis-GMA	1565-94-2	45	**4.94**	AOO	NT	1.57–50	7.4	4.3	**3.8**	**7.2**		3.54	3.4	4.35	P	P1	P3	N1
Tridecane	629-50-5	48.4	**6.73**	AOO	NT	25–100	3.6	1.3	1.7	**4.4**				87.44	P	N.D.	N.D.	N.D.
Limonene	5989-27-5	69	**4.83**	AOO	3.51	0.39–3.13	**98.7**	2.4	**2.8**	**12**	1.2	1.51	0.92	1.75	P	P3	N3	P3
1-Butanol	71-36-3	–	0.84	AOO	3.75	1.56–6.25	2.3	1.0	1.1	0.9					N	N1	N2	N1
4-Hydroxybenzoic acid	99-96-7	–	1.39	AOO	2.42	0.39–3.13	6.5	1.5	1.3	2.6					N	N1	N2	N1
Acetanisole	100-06-1	–	1.75	AOO	0.69	0.20–1.56	3.4	1.0	1.7	1.4					N	N1	P2	N1
Benzalkonium chloride	8001-54-5	–	2.93	AOO	0.44	0.05–0.78	1.8	1.2	1.0	**6.2**				0.1	P	N1	N2	N1
Benzylbutylphthlate	85-68-7	–	**4.84**	AOO	NT	25–100	1.0	1.3	1.2	1.3					N	N1	P3	N1
Dibutyl phthalate	84-74-2	–	**4.61**	AOO	NT	25–100	3.5	1.98	1.2	2.6					N	N1	P3	N1
Glycerol[f]	56-81-5	–	−1.65	DW	NT	25–100	12.3	0.8	0.7	3.1					N	N1	N2	N1
Cetyltrimethylammonium Bromide	57-09-0	–	3.18	50%EtOH	1.28	0.20–1.56	1.7	1.1	1.0	3.1					N	N3	N.D.	N.D.
Hexane	110-54-3	–	3.29	AOO	60.3	25–100	9.9	1.1	1.9	1.9					N	N2	N2	N4
Isopropanol	67-63-0	–	0.28	AOO	32.1	6.25–50	4.3	1.0	1.1	1.6					N	N1	N2	N1
Lactic acid[f]	50-21-5	–	−0.65	DW	3.8#	1.56–6.25	1.0	1.0	1.1	0.8					N	N1	N2	N1
Methyl salicylate	119-36-8	–	2.6	AOO	2.39	0.39–3.13	3.6	1.3	1.6	**6.0**				0.72	P	N1	N2	N1
Octanoic acid	124-07-2	–	3.03	AOO	0.88	0.20–1.56	**38.1**	3.5	1.1	**5.9**	0.5	0.52		0.63	P	N1	N2	P1
Propylene glycol	57-55-6	–	−0.78	DW	NT	25–100	13.7	1.8	0.9	0.9					N	N1	N2	N1
Salicylic acid[f]	69-72-7	–	2.24	AOO	2.19#	0.39–3.13	11.0	1.9	1.6	2.1					N	P2	N2	P3
Sodium lauryl sulfate[f]	151-21-3	–	1.69	DW	1.07	0.31–2.5	5.2	1.1	0.9	**8.6**				0.37	P	P1	N2	N1
Sulfanilamide	63-74-1	–	−0.55	50%EtOH	>5.0	1.25–5	0.3	1.1	0.9	0.8					N	N1	N2	N1
Vanillin	121-33-5	–	1.05	AOO	1.45	0.39–3.13	4.4	1.1	1.4	1.6					N	P1	N2	N1

[a] Calculated by KOWWIN ver.1.68 in EPI suite™. The values exceeding logKow of 3.5 are indicated in bold.
[b] NT indicated "Non-Toxic", which means cell viability were over 80% at tested concentration of 100%. [#]Based on the data from MTT assay.
[c] Mean values of triplicates are shown. The values exceeding the cut-off value of each marker gene (ATF3, 15-fold; DNAJB4 and GCLM, 2-fold; and IL-8, 4-fold induction) are indicated in bold.
[d] If the test chemical exceeded the cut-off value of at least one out of the four marker genes, the chemical was judged as positive (P). If not, the chemical was judged as negative (N).
[e] For data from in vitro tests, chemicals judged as positive are indicated as "P", and those judged as negative chemicals are indicated as "N" in the Table. Data sources of these test results (1. Natsch et al., 2013; 2. Takenouchi et al., 2015; 3. Urbisch et al., 2015;4. Jaworska et al., 2015) are also shown. "N.D" in the table indicates "No Data".
[f] ECVAM reference chemical (Casati et al., 2009).
[g] Putative pre/pro-hapten (Urbisch et al., 2015).

2015a, 2015b). However, since all of these tests are performed in an aqueous system (buffer or culture medium), it is often difficult to correctly evaluate substances with high lipophilicity (i.e. with logKow >3) (OECD, 2012b) due to their precipitation or phase separation (Takenouchi et al., 2013; Natsch et al., 2013). It is also difficult to evaluate pre/pro-haptens, which form protein reactive haptens through either air-oxidation or metabolic conversion (Aptula et al., 2007), due to the limited metabolic systems in the assays (OECD, 2015a, 2015b and OECD, 2015C). Thus, to fully replace in vivo skin sensitization tests, a novel test that can evaluate lipophilic chemicals and pre/pro-haptens is necessary.

To overcome the above assay limitations, a three dimensional reconstructed human epidermis model (RhE model), which consists of normal human-derived epidermal keratinocytes (NHEKs), could be a useful tool. Since RhE models are cultured at an air–liquid interface, test chemicals are directly applicable to RhE models in a manner similar to real skin, suggesting that lipophilic chemicals could be applied and tested correctly. Moreover, it has been reported that RhE models exhibit metabolic capability similar to that of human skin (Luu-The et al., 2009; Götz et al., 2012a, 2012b; Eilstein et al., 2014), suggesting that RhE models would successfully metabolize pre/pro-haptens for correct evaluation. On the other hand, mechanistically relevant biomarkers that reflect keratinocyte responses in the early phase of skin sensitization are necessary in RhE-based assays. The two main important keratinocyte responses that occur in the skin sensitization AOP are; 1) Inflammatory response (e.g. activation of inflammatory cytokines) and 2) Induction of cytoprotective gene pathways (e.g. antioxidant/electrophile response element (ARE/EpRE)-dependent pathways) (OECD, 2012a). Key molecules that regulate the two pathways, $P2X_7$, a purinergic receptor involved in the production of pro-inflammatory cytokines such as IL-1β and IL-18 (Kaplan et al., 2012), and nuclear factor E2-related factor 2 (Nrf2), a transcription factor that is involved in the activation of antioxidant and cytoprotective genes by binding to ARE (Uruno and Motohashi, 2011), have been reported to modulate skin sensitization in vivo either positively ($P2X_7$) or negatively (Nrf2) (Weber et al., 2010; et al. et al., 2013).

Based on these data, some RhE-based skin sensitization assays that are focused on the skin sensitization AOP have been developed and reported by several research groups: The Epidermal equivalent (EE) assay is based on the production of inflammatory cytokines such as IL-18 in multiple RhE models such as epiCS® (Gibbs et al., 2013); the SenCeeTox® assay is mainly based on eight Nrf2-dependent genes in the EpiDerm™ and SkinEthicRHE assays (McKim et al., 2012); and the SENS-IS assay focuses on 24 Nrf2-dependent genes and 41 additional genes (SENS-IS genes) in the EpiSkin assay (Cottrez et al., 2016). The biomarkers of these assays such as IL-18 and Nrf2 dependent genes are closely related to keratinocyte responses in the skin sensitization AOP (OECD, 2012a), which strongly supported the idea that these assays could be mechanistically relevant for evaluation of skin sensitization. However, the EE assay uses only one biomarker, IL-18, which is related to inflammatory responses, and the SenCeeTox® assay relies on Nrf2-dependent genes that are related to cytoprotective responses, implying that these assays have lower sensitivity for sensitizers that cannot induce IL-18 production or activate the Nrf2 pathway. Also, the capability of these two assays to predict the skin sensitization potential of lipophilic chemicals as well as of pre/pro-haptens has yet to be fully assessed. On the other hand, the SENS-IS assay can detect both inflammatory and cytoprotective responses by using 65 marker genes and showed 100% accuracy for 150 chemicals when compared to the LLNA (Cottrez et al., 2016). However, the use of large sets of genes would complicate an assay system (e.g. procedure and prediction) and lacks transparency in the rationale used for interpretation for a prediction.

We have developed a novel skin sensitization assay, termed "EpiSensA", that is based on the expression of marker genes related to cellular stress responses in an RhE model; EpiDerm™ (Saito et al., 2013a). Our previous study (Saito et al., 2013a) demonstrated that three marker genes, ATF3, DNAJB4 and GCLM, in the EpiSensA could correctly predict the skin sensitization potential of 16 reference chemicals recommended by the European Centre for the Validation of Alternative Methods (ECVAM) (Casati et al., 2009). The 16 chemicals included four pre/pro-haptens (e.g. eugenol), suggesting that these three marker genes would be useful for evaluation of the skin sensitization potential of chemicals including pre/pro-haptens. Of the three marker genes in the EpiSensA, GCLM has been reported to be regulated through the Nrf2/ARE pathway in the HaCaT human keratinocyte cell line (MacLeod et al., 2009), suggesting that it is a mechanistically relevant marker for the prediction of skin sensitization. Also, it has been suggested that DNAJB4 might be related to a "cytoprotective response" based on its original function (i.e. a molecular chaperone) (Qiu et al., 2006), whereas up-regulation of DNAJB4 by sensitizers has been reported to be independent of Nrf2 (Emter et al., 2013). On the other hand, ATF3 might be involved in inflammatory pathways based on the previous finding that the expression of ATF3 in NHEKs was up-regulated by ATP (Ohara et al., 2010), which is sensed through $P2X_7$ and which modulates the "inflammatory pathway" in the early phase of skin sensitization (Kaplan et al., 2012). Based on the combined data, it is expected that the EpiSensA would have higher sensitivity in predicting skin sensitization due to the fact that it has gene markers that reflect two different keratinocyte responses (inflammatory and cytoprotective). However, there has been no direct evidence that ATF3 and DNAJB4 are involved in keratinocyte responses in the skin sensitization AOP.

The activation of inflammatory cytokines has also been shown to play an important part in the inflammatory response of keratinocytes (OECD, 2012a). An earlier study that used several sensitizers and irritants suggested that IL-8, which is a chemokine that promotes the recruitment of polymorphonuclear leukocytes in the initiation of cutaneous inflammation (Barker et al., 1991), is an indicator of skin sensitization in a RhE model (Coquette et al., 2003). Recently, several investigators demonstrated that IL-8 could be a good skin sensitization marker in human keratinocytes (McKim et al., 2010; Bae et al., 2015). Although all of the above studies used dozens of test chemicals with relatively high water solubility, it is tempting to hypothesize that IL-8 would also be a good biomarker of skin sensitization for lipophilic chemicals in an RhE model.

In this study, in order to further support the mechanistic relevance of the EpiSensA marker genes to predict skin sensitization, we first determined whether the up-regulation of four marker genes (ATF3, IL-8, DNAJB4, GCLM) in NHEKs is regulated by two important molecules, $P2X_7$ and Nrf2, that are involved in key keratinocyte responses of inflammation and cytoprotection, respectively. We then examined the utility of the four marker genes for predicting the skin sensitization potential and potency of 29 chemicals with relatively high lipophilicity (logKow ≥ 3.5) as well as of 43 chemicals with relatively low lipophilicity (logKow < 3.5) including 11 pre/pro-haptens in a RhE model to assess whether the EpiSensA can overcome the limitations of existing in vitro tests.

2. Materials and methods

2.1. Chemicals and sample preparation

For *Assessment of the regulation mechanism of marker genes in NHEKs* (Section 2.2), a representative sensitizer 2,4-dinitrochlorobenzene (DNCB) purchased from Sigma-Aldrich (St. Louis, MO, USA) was evaluated. DNCB was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). For *Assessment of the predictive performance of marker genes* (Section 2.3), a total of 72 test chemicals that are shown in Table 1 were selected based on their skin sensitizing properties reported in the literature (Gerberick et al., 1992; Gerberick et al., 2005; Kimber et al., 1998 and Kern et al., 2010), their diverse chemical structures, and their availability from commercial sources. This collection includes 29 chemicals with logKow ≥ 3.5 ("lipophilic chemicals" in this study),

which have been reported to show a relatively high false negative ratio in the h-CLAT (Takenouchi et al., 2013), as well as 43 chemicals with logKow < 3.5 ("hydrophilic chemicals" in this study). In addition, 11 putative pre/pro-haptens were also included based on the previous literature referred to in Urbisch et al., 2015. Most of the tested chemicals were purchased from Sigma–Aldrich, except for tetrachlorosalicylanilide (TCSA; Kanto Chemical Co. Inc., Tokyo, Japan), methyldibromoglutaronitrile (Alfa Aesar Ward Hill, MA, USA), lillial and undecylenic acid (Wako Pure Chemicals, Osaka, Japan), and 2-ethylhexyl acrylate (EHA; Nacalai Tesque, Kyoto, Japan). Tested chemicals were dissolved in AOO (acetone (Sigma-Aldrich):olive oil (Kanto Chemical) = 4:1), distilled water (DW; Otsuka Pharmaceutical Factory, Inc., Tokyo, Japan) or 50% ethanol in DW (50% EtOH; ethanol was purchased from Kanto Chemical) when applied to the RhE model, since all of these vehicles have often been adopted in dermal application in animal testings and failed to affect cytotoxicity under the testing condition used in this study. The CAS No., LLNA Estimated Concentration of a test substance needed to produce a stimulation index of three (EC3) values (%) (OECD, 2010), logKow calculated by KOWWIN ver.1.68 in EPI suite™ (Environmental Protection Agency, Washington, DC, USA), and vehicle for each chemical are listed in Table 1.

2.2. Assessment of the regulation mechanism of marker genes in NHEKs

2.2.1. Cell culture

NHEKs (KURABO, Osaka, Japan) were cultured in HuMedia–KG2 (KURABO) supplemented with insulin, bovine pituitary extract, epidermal growth factor, hydrocortisone, kanamycin, and amphotericin B. When used for experiments, cells were seeded in 12-well plates at a density of 1.0×10^5 cells per well in 1 mL of culture medium.

2.2.2. siRNA experiment

The transfection mix was prepared by mixing 940 μL of culture medium, 0.2 μL of 10 μM control siRNA (sc-37007, Santa Cruz Biotech, Santa Cruz, CA, US) or Nrf2-siRNA (sc-37030, Santa Cruz Biotech) (final concentration, 5 nM) and 60 μL of Hiperfect transfection reagent (Qiagen, Valencia, CA, USA). This transfection mix was incubated at room temperature for 10 min. Subsequently, the cell culture medium of NHEKs that were pre-seeded in 12-well plates overnight was exchanged for 1 mL of the transfection mix. Twenty four hours later, the cells were washed with 1 mL of HEPES buffer (Kurabo) and exposed to vehicle (DMSO; final concentration, 0.2%) or DNCB (2.2 μg/mL) in 1 mL of culture medium for 6 h in triplicate.

2.2.3. $P2X_7$ receptor blocking experiment

For blocking the $P2X_7$ receptor, KN-62 (Santa Cruz Biotech), a human selective $P2X_7$ antagonist (Anderson and Nedergaard, 2006) was used. KN-62 dissolved in DMSO was diluted in culture medium to a concentration of 30 μM and was then applied to NHEK cells in 12-well plates. Fifteen minutes later, the cells were washed with 1 mL of HEPES buffer and exposed to the vehicle (DMSO; final concentration, 0.2%) or DNCB (2.2 μg/mL) in 1 mL of culture medium for 6 h in triplicate.

2.2.4. RNA isolation

Following 6 h of treatment with chemicals, the cells were lysed with RLT buffer (Qiagen) containing 2.5% DL-Dithiothreitol (Sigma-Aldrich). The RNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and was then quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and stored at −80 °C until use.

2.2.5. cDNA synthesis

The Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) was used to prepare cDNA. The mixture consisted of 1 μL of a 10 mM dNTP mix, 1 μL of Oligo (dT) (0.5 μg/μL), RNase free water (the above 3 reagents were supplied by the manufacturer) and 0.5 μg of total RNA (variable volume) was added to achieve a total volume of 10 μL. The mixture was incubated at 65 °C for 5 min and then on ice for 1 min. A mixture of 10× RT Buffer (2 μL), 25 mM $MgCl_2$ (4 μL), 0.1 M DTT (2 μL), and RNase Out (1 μL) was added to the reaction and incubated at 42 °C for 2 min. Subsequently, 1 μL of Super Script III was added and incubated at 50 °C for 50 min. The reaction was terminated by incubation at 85 °C for 5 min. The cDNA was treated with 1 μL RNase H for 20 min at 37 °C and was then stored at −20 °C.

2.2.6. Real-time PCR

The primers and probes for five genes: activating transcription factor 3 (ATF3); DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4); glutamate-cysteine ligase, modifier subunit (GCLM); interleukin-8 (IL-8); and Nrf2, and for one endogenous control gene (glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) were designed by Assays-by-Design Service from Applied Biosystems and the sequence information remains confidential. The primers and probes were delivered as a 20× Taqman Gene Expression Assay mix (Applied Biosystems). A total volume of 20 μL sample, which consisted of 1 μL TaqMan® Gene Expression Assay 20×, 10 μL TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μL cDNA template and 8 μL dH_2O was prepared and applied to an optical reaction plate (96-well plate; Applied Biosystems). Real-time PCR reactions were performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). Relative gene expression levels versus control (fold change) were calculated using the $2^{-\Delta\Delta Ct}$ method (comparative CT method; Livak and Schmittgen, 2001).

2.2.7. Statistics

Student's *t*-test was used to evaluate statistical significance. *p*-Values < 0.01 were considered to be statistically significant.

2.3. Assessment of the predictive performance of marker genes for 72 chemicals

2.3.1. Tissue culture

The RhE model "LabCyte EPI-MODEL (24 well format)" (Japan tissue Engineering Co. Ltd., Aichi, Japan) was used in this study. This model consists of NHEKs whose biological origins are neonatal foreskins, and which construct a multilayer structure consisting of a fully differentiated epithelium with features of a normal human epidermis, including a stratum corneum (Katoh et al., 2009). This RhE model was adopted in the OECD test guideline 439; In vitro Skin Irritation: Reconstructed Human Epidermis Test Method (OECD, 2013). The tissues were pre-cultured overnight at 37 °C (5% CO_2) in 0.5 mL/tissue of culture media provided by the manufacturer.

2.3.2. Chemical exposure

For dose finding, solid test chemicals were dissolved in an appropriate vehicle (AOO, DW or 50% EtOH) at the maximum soluble concentration (from 50%) and 4-fold serial dilutions were then made to prepare test the chemical solutions at doses ranging from the maximum soluble concentration to a minimum concentration of 0.02%. Liquid test chemicals were serially diluted 4-fold with an appropriate vehicle to prepare test chemical solutions at doses ranging from 100% (neat chemical) to a minimum dose of 0.02%. A 5 μL aliquot of each test chemical solution was applied to the surface of the LabCyte EPI-MODEL (1 well per group) and was incubated for 6 h at 37 °C (5% CO_2). An untreated and a killed control tissue exposed to 10 μL of 10% triton (1 well per group) were prepared as control tissues for cell viability measurement. Cell viability was assessed using the lactate dehydrogenase assay (LDH assay) (see

Section 2.3.4).For the main study, each test chemical was dissolved at the minimum concentration that showed <80% cell viability in the dose finding study. The test chemical solutions were prepared by making 2-fold serial dilutions of the maximum concentration that showed >90% cell viability in the dose finding study. Generally, four to five different concentrations were prepared for each test chemical. If no cytotoxicity was observed in the dose finding study, at least three test chemical solutions of different concentrations (e.g. 1×, 1/2× and $1/2^2$× maximum soluble concentration) were prepared. A 5 μL aliquot of each test chemical solution was applied to the surface of the LabCyte EPI-MODEL (3 wells per group) and was incubated for 6 h at 37 °C (5% CO_2). An untreated tissue, a killed control tissue exposed to 10 μL of 10% triton, and vehicle-treated tissues (3 wells per group) were prepared as control tissues.

2.3.3. RNA isolation, cDNA synthesis and Real-time PCR

Following 6 h treatment with chemicals, the tissue surfaces were rinsed three times in 500 μL pre-warmed D-PBS(−) (Life Technologies, Carlsbad, CA, USA). The tissues were then gently removed, placed into a 1.5 mL microtube containing 0.5 mL TRIzol® (Invitrogen) and homogenized by vortex mixing. Chloroform (100 μL; Tokyo Chemical Industry, Tokyo, Japan) was added to each microtube containing a homogenized sample. The samples were centrifuged at 12,000 ×*g* for 15 min at 4 °C. The aqueous phase containing RNA was transferred to a 1.5 mL microtube and RNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA was quantified using a ND-1000 spectrophotometer and was stored at −80 °C until use. cDNA synthesis and real-time PCR were performed in accordance with the protocol described in Sections 2.2.5 and 2.2.6.

2.3.4. LDH assay

To determine cell viability, LDH activity in the culture media was measured using an LDH cytotoxicity detection kit (Takara Bio, Inc., Tokyo, Japan) in accordance with the manufacturer's instructions. Upon LDH release, this kit creates a red formazan dye that absorbs light at 490 nm. The absorbance of each well was measured at 490 nm and 620 nm (the reference wavelength) using a plate reader (BMG LABTECH GmbH, Offenburg, Germany). ΔAbsorbance was calculated by subtracting the absorbance at 620 nm from the absorbance at 490 nm. Media from an untreated tissue or a tissue treated with 10% Triton X-100 was used for calculation of minimal LDH release (negative control) or maximal LDH release (killed control), respectively. Cell viability was calculated using the following formula:

$$\text{Cell viability}\ (\%) = 100-(\Delta\text{Absorbance of negative control}-\Delta\text{Absorbance of a chemical}-\text{treated group})/(\Delta\text{Absorbance of killed control}-\Delta\text{Absorbance of negative control})\times 100$$

For chemicals that are known to affect the LDH assay (e.g. lactic acid, salicylic acid), the MTT assay was alternatively performed (see Section 2.3.5).

2.3.5. MTT assay

A methylthiazolydiphenyl-tetrazolium bromide (MTT, Sigma-Aldrich Co.) solution was prepared at a concentration of 0.5 mg/mL in culture medium (provided by Japan tissue Engineering Co. Ltd). The tissue surfaces were washed three times with D-PBS (−) and 300 μL of MTT solution was placed on top and incubated for 3 h at 37 °C (5% CO_2). After incubation, the MTT solution was discarded and the tissues were gently removed and placed into 200 μL isopropanol (Sigma-Aldrich). The extraction process was performed for 2 h at room temperature. The absorbance of the extract was measured at 570 nm using a plate reader (BMG LABTECH GmbH, Offenburg, Germany). The absorbance of the untreated control tissue exposed to culture media was set to represent 100% cell viability and the results are expressed as percentage of an untreated control.

$$\text{Cell viability}\ (\%) = \text{Absorbance of chemical}-\text{treated tissues}/\text{Absorbance of untreated tissues}\times 100$$

2.3.6. Data analysis and prediction

The mean value (3 wells per group) of maximal fold induction (I_{max}) of each gene was determined using data obtained from chemical concentrations that displayed over 80% cell viability. When the I_{max} of at least one out of the four marker genes exceeded the cut-off value of that gene (15-fold for ATF3, 2-fold for DNAJB4 and GCLM, and 4-fold for IL-8) at over 80% cell viability, the chemical was judged as positive (The EpiSensA prediction). Also, the estimated concentration (EC) that showed a fold induction of each cut-off value (ATF3 EC15, DNAJB4 EC2, GCLM EC2 and IL-8 EC4) at over 80% cell viability was calculated using linear interpolation from the values above and below the induction thresholds. If fold inductions at all tested concentrations exceeded the cut-off values, the EC values were calculated by linear extrapolation from the values at the minimum two tested concentrations.

3. Results

3.1. Assessment of the regulation mechanism of marker genes in NHEKs

We first checked the involvement of Nrf2 in the up-regulation of DNAJB4 and GCLM by a representative sensitizer, DNCB, in NHEKs. To this end, Nrf2 expression was inhibited by knock-down of Nrf2 with siRNA. The up-regulation of DNAJB4 and GCLM by DNCB was significantly suppressed (67% inhibition of DNAJB4 and 64% inhibition of GCLM) when Nrf2 expression was strongly down-regulated (<10% compared with the normal condition) (Fig. 1A). On the other hand, the up-regulation of ATF3 and IL-8 DNCB was not significantly inhibited by Nrf2 siRNA treatment (Fig. 1B). The induction level of ATF3 by DNCB was 1.9-fold higher under the Nrf2 knock-down condition, but there's no significant difference ($p = 0.02$). These results suggested that the up-regulation of DNAJB4 and GCLM by sensitizers could be partly regulated by Nrf2. We next checked the involvement of $P2X_7$ in the up-regulation of ATF3 and IL-8 by DNCB, by blocking $P2X_7$ with the $P2X_7$ specific antagonist KN-62. The up-regulation of both ATF3 and IL-8 by DNCB was significantly inhibited by KN-62 (64% and 77% inhibition, respectively) (Fig. 2A), whereas the up-regulation of DNAJB4 and GCLM was not (Fig. 2B). These results indicated that $P2X_7$ could be partly involved in the up-regulation of ATF3 and IL-8 by sensitizers in NHEKs. These combined results indicated that the up-regulation of the EpiSensA marker genes by sensitizers in NHEKs could be regulated through the key molecules $P2X_7$ or Nrf2 in the skin sensitization AOP.

3.2. Predictive performance of marker genes to 72 chemicals

Next, we evaluated the utility of the four marker genes, ATF3, DNAJB4, GCLM and IL-8, for prediction of the skin sensitization potential of chemicals. A total of 72 chemicals (43 hydrophilic chemicals; logKow < 3.5, and 29 lipophilic chemicals; logKow ≥ 3.5) were evaluated using the EpiSensA protocol described in *Materials & Methods* (Section 2.3). Fig. 3 shows the fold induction of the four marker genes (vs. vehicle control) and the cell viabilities calculated for four of the lipophilic sensitizers: bisphenol A diglycidyl ether; BADGE; undec-10-enal, EHA; and abietic acid. Each sensitizer up-regulated the expression of marker genes at non- to slightly toxic concentrations (cell viability was >80%). The induction levels of four genes were dose-dependently increased by each of the four lipophilic sensitizers. The maximum induction levels of ATF3 (ranging from 40.4-fold for BADGE to 174-fold for EHA) and IL-8 (ranging

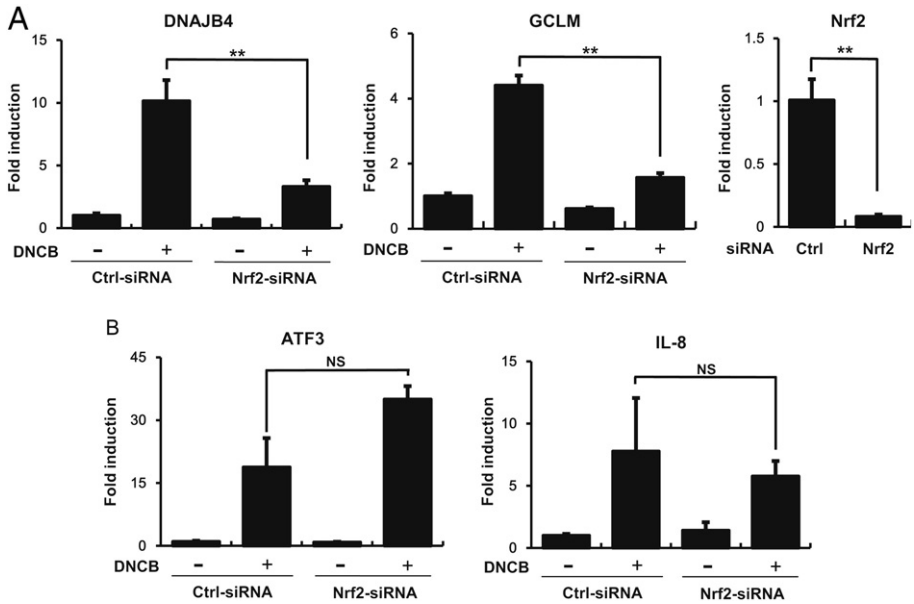

Fig. 1. The effect of Nrf2 knockdown on the up-regulation of DNAJB4 and GCLM genes by DNCB. NHEKs were transfected with Control- (Ctrl-) or Nrf2-siRNA (5 nM) for 24 h, followed by their exposure to vehicle (DMSO) or DNCB (2.2 μg/mL) for 6 h. The expression levels of DNAJB4, GCLM and Nrf2 genes (A), and ATF3 and IL-8 genes (B) were measured using real-time PCR. Data are shown as means ± SD values (3 wells per group). Statistical analyses were performed using Student's *t*-test. $^{**}p < 0.01$. Results are representative of at least two independent experiments.

from 25.3-fold for EHA to 115.7-fold for undec-10-enal) at over 80% cell viability tended to be higher than the maximum induction levels of DNAJB4 (ranging from 2.8-fold for Abietic acid to 83.9-fold for EHA) and GCLM (ranging from 3.0-fold for undec-10-enal to 28.9-fold for EHA).

Fig. 4 shows the fold induction of the four marker genes (vs. vehicle control) and cell viabilities of the cells treated with each of the four pre/pro-haptens: p-phenylenediamine (PPD); metol; ethylenediamine; and eugenol. As observed with the lipophilic sensitizers, each pre/pro-hapten up-regulated the expression of marker genes at non- to slightly toxic concentrations. Dose response patterns of ATF3 and IL-8 were similar for the four pre/pro-haptens, whereas the induction levels of ATF3 were higher than those of IL-8. On the other hand, the dose response patterns of DNAJB4 and GCLM were similar for three of the pre/pro-haptens (PPD, metol and eugenol), but were different for ethylene diamine (Fig. 4). Also, PPD, metol and eugenol induced higher up-regulation of

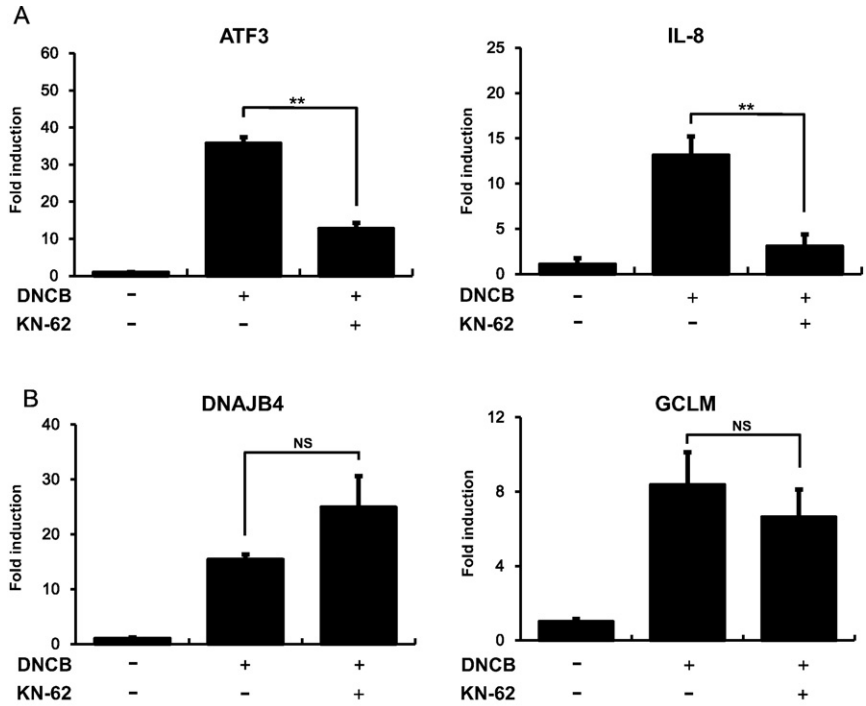

Fig. 2. The effect of $P2X_7$ blockade on the up-regulation of ATF3 and IL-8 genes by DNCB. NHEK cells were treated with vehicle (DMSO) or the $P2X_7$ antagonist KN-62 (30 μM) for 15 min, followed by their exposure to vehicle (DMSO) or DNCB (2.2 μg/mL) for 6 h. The expression levels of ATF3 and IL-8 genes (A), and DNAJB4 and GCLM genes (B) were measured using real-time PCR. Data are shown as means ± SD values (3 wells per group). Statistical analyses were performed using Student's *t*-test. $^{**}p < 0.01$. Results are representative of at least two independent experiments.

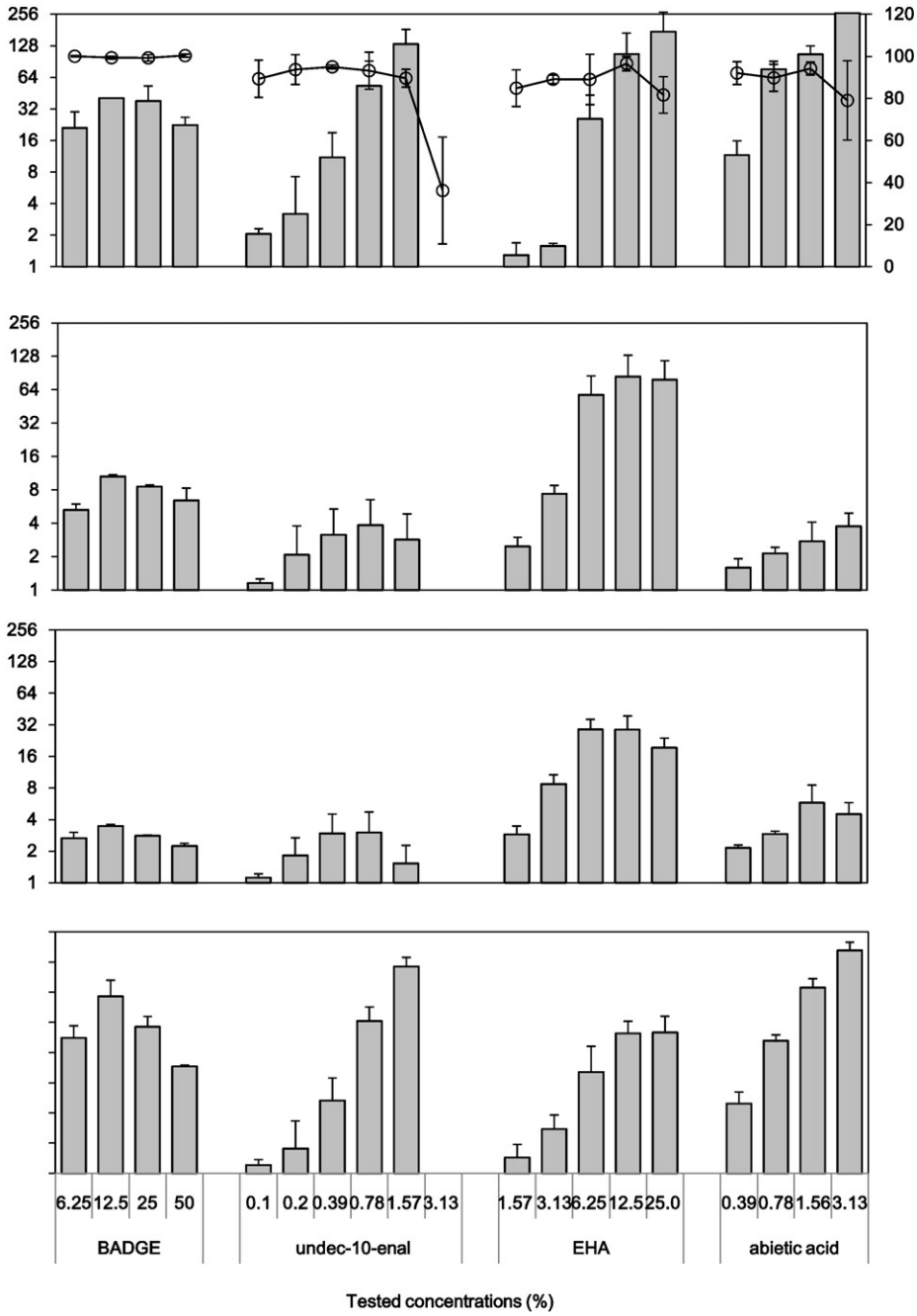

Fig. 3. The up-regulation of four marker genes by lipophilic sensitizers. The LabCyte EPI-MODEL was treated with vehicle (AOO) or with each lipophilic sensitizer (BADGE, undec-10-enal, EHA or abietic acid) at the indicated concentrations (%) for 6 h. Cell viability (A; white circles) was assessed using the LDH assay. The expression levels of four marker genes were analyzed using real-time PCR. Fold induction of each marker gene (A, ATF3; B, DNAJB4; C, GCLM; and D, IL-8; gray bars) was calculated compared to AOO-treated tissues. Data are shown as means ± SD values (3 wells per group).

the expression of DNAJB4 and GCLM compared to the up-regulation of ATF3 and IL-8 at over 90% cell viability. These results suggested that the four marker genes could have the potential to detect lipophilic skin sensitizers as well as pre/pro-haptens. Additionally, up-regulation of inflammatory genes (ATF3 and IL-8), or cytoprotective genes (DNAJB4 and GCLM) would contribute more strongly to the detection of lipophilic sensitizers or pre/pro-haptens, respectively.

Table 1 shows the IC20 (inhibitory concentration showing 20% decrease of cell viability), applied dose ranges in main study, I_{max} and EC values of the four marker genes, and the EpiSensA prediction (positive, P, or negative, N, based on the prediction model described in Section 2.3.5) for the 72 test chemicals. The I_{max} values of the four marker genes, which depends on the test chemicals, varied from 0.30 (sulfanilamide) to 704.8 (pentachlorophenol) for ATF3, from 0.8 (glycerol) to 202.7 (damascone) for DNAJB4, from 0.7 (glycerol) to 49.5 (damascone) for GCLM and from 0.8 (LA and sulfanilamide) to 147.1 (farnesol) for IL-8, regardless of their lipophilicity. Forty eight out of the 54 tested sensitizers showed an I_{max} of over 4-fold for ATF3, which was a cut-off value set in our initial study using EpiDerm™ (Saito et al., 2013a). However, 9 out of the 18 tested non-sensitizers were also observed to have an I_{max} of over 4-fold for ATF3; these non-sensitizers included non-sensitizing, non-irritating chemicals such as glycerol (I_{max} of 12.3-fold) and propylene glycol (I_{max} of 13.7-fold). On the other hand, 38 out of the 54 tested sensitizers showed an I_{max} of over 2-fold for DNAJB4 whereas 17 of the tested non-sensitizers, except for octanoic acid (OA), did not.

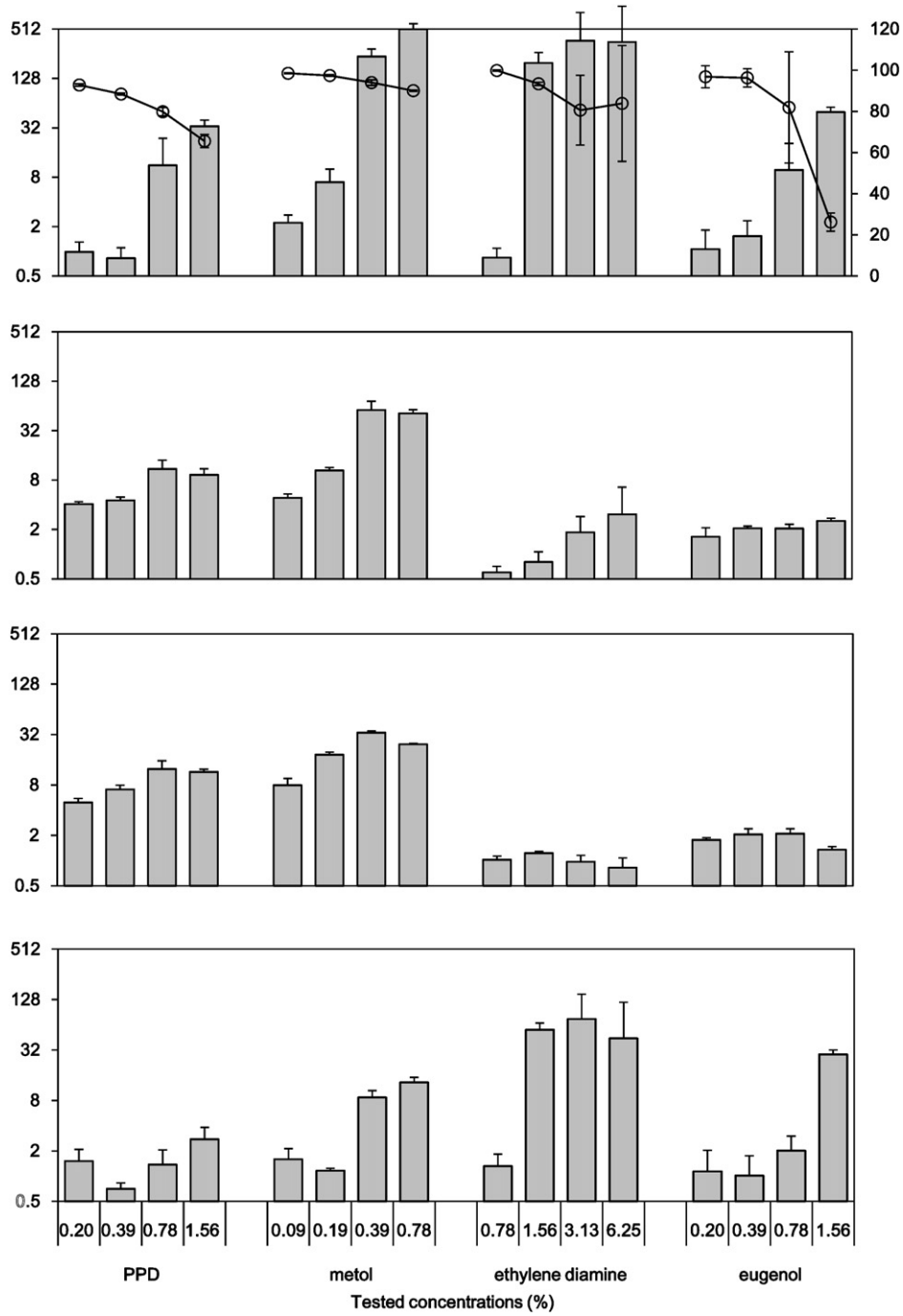

Fig. 4. The up-regulation of four marker genes by pre/pro-haptens. The LabCyte EPI-MODEL was treated with vehicle (AOO or DW) or with each pre/pro-hapten (PPD, metol, ethylene diamine or eugenol) at the indicated concentrations (%) for 6 h. Cell viability (A; white circles) was assessed using the LDH assay. The expression levels of four marker genes were analyzed using real-time PCR. Fold induction of each marker gene (A, ATF3; B, DNAJB4; C, GCLM; and D, IL-8; gray bars) was calculated compared to AOO or DW-treated tissues. Data are shown as means ± SD values (3 wells per group).

Also, 26 of the tested sensitizers, but none of the tested non-sensitizers, showed an I_{max} of over 2-fold for GCLM. Regarding IL-8, which was a newly added marker gene, 41 of the tested sensitizers showed an I_{max} of over 4-fold, whereas 14 out of the 18 tested non-sensitizers did not. Three irritants; benzalkonium chloride (BKC); methyl salicylate (MS) and sodium dodecyl sulfate (SLS); as well as octanoic acid (OA), also showed an I_{max} of >4-fold, although the values were relatively low (<10-fold).

We then determined the cut-off value of each marker gene based on the following criteria; 1) best predictive performance (sensitivity, specificity and accuracy) when a test chemical was judged as positive for at least one out of two genes related to inflammatory responses (ATF3 and IL-8), or cytoprotective responses (DNAJB4 and GCLM), and 2) over 2-fold induction (to ensure the reliability of gene expression data by Real-time PCR). Regarding the inflammatory response genes, a 15-fold increase in ATF3 and a 4-fold increase in IL-8 provided the best predictive performance of sensitivity (82%), specificity (78%) and accuracy (81%). On the other hand, regarding cytoprotective genes, a 2-fold increase in both DNAJB4 and GCLM, which was the same value as that in our initial study (Saito et al., 2013a), showed the best predictive performance (sensitivity (72%), specificity (94%) and accuracy (78%)) (Table 2). In both cases, the

Table 2
Predictive performance of the EpiSensA as well as of each marker gene.

	ATF3	IL-8	Inflammatory (Inflam.)	DNAJB4	GCLM	Cytoprotective (Cyto.)	EpiSensA
Positive criteria	15-fold	4-fold	Positive in ATF3 or IL-8	2-fold	2-fold	Positive in DNAJB4 or GCLM	Positive in Inflam. or Cyto.
A. Lipophilic chemicals (29 chemicals)							
Sensitivity (%)	67	81	85	67	37	67	93
Specificity (%)	100	100	100	100	100	100	100
Accuracy (%)	69	83	86	69	41	69	93
B. Hydrophilic chemicals (43 chemicals)							
Sensitivity (%)	63	70	74	74	59	78	96
Specificity (%)	94	75	75	94	100	94	75
Accuracy (%)	74	72	74	81	74	84	88
C. Pre/pro-haptens (11 sensitizers)[a]							
Sensitivity (%)	45	55	64	82	55	91	100
Specificity (%)	–	–	–	–	–	–	–
Accuracy (%)	45	55	64	82	55	91	100
D. Overall (72 chemicals)							
Sensitivity (%)	65	76	81	70	48	72	94
Specificity (%)	94	78	78	94	100	94	78
Accuracy (%)	72	76	81	76	61	78	90

Predictive performances for 29 lipophilic (logKow ≥ 3.5), 43 hydrophilic (logKow < 3.5), 11 pre/pro-haptens and the total 72 chemicals are shown.
[a] These chemicals included both hydrophilic and lipophilic sensitizers.

combination of two marker genes (ATF3 and IL-8, or DNAJB4 and GCLM) showed better sensitivity and accuracy than a single marker gene in each keratinocyte response (Table 2). Also, the predictive performance of inflammatory genes (ATF3 and IL-8) to lipophilic chemicals was higher (86% accuracy) than that of cytoprotective genes (DNAJB4 and GCLM), whereas the predictivity for pre/prohaptens was higher (91% accuracy) for cytoprotective genes than for inflammatory genes.

Finally, when each test chemical was judged as positive if at least 1 marker gene related to inflammatory or cytoprotective responses provided positive results (EpiSensA prediction model), excellent predictive values of sensitivity (over 90%) and accuracy (approximately 90%) were obtained for the 72 test chemicals including for the lipophilic chemicals and the pre/pro-haptens (Table 2). These data suggested that the combination of four genes related to two keratinocyte responses could achieve higher sensitivity and accuracy in predicting the skin sensitization potential of broad sets of chemicals.

We also compared the predictive performance of the EpiSensA with those of three existing in vitro tests (the DPRA, KeratinoSens™ and h-CLAT). Table 1 shows that 11 tested lipophilic sensitizers (logKow ≥ 3.5) including dibutyl aniline, which were judged as false negative in at least two existing in vitro tests, provided positive results in the EpiSensA (Table 1). In addition, three hydrophilic sensitizers (benzoyl peroxide, diethylene triamine and resorcinol; logKow < 3.5), which could not be detected in at least two existing in vitro tests, were also correctly judged as sensitizers in the EpiSensA (Table 1). The sensitivity, specificity and accuracy of the four in vitro tests are shown in Table 3. Regarding lipophilic chemicals, the EpiSensA showed much better sensitivity and accuracy (93%) than the other three in vitro tests (ranging from 44% to 67%). Furthermore, the sensitivity of the EpiSensA to hydrophilic chemicals (96%) or pre/pro-haptens (100%) was greater compared to the other in vitro tests (ranging from 70 to 81%, or 55 to 80%, respectively) (Table 3). Finally, the sensitivity (94%) and accuracy (90%) of the EpiSensA for the 72 tested chemicals were better than those of the other three tests (Table 3).

We then assessed the potency predictive capacity of the EpiSensA based on the EC values of the four marker genes shown in Table 1. Fig. 5 shows scatter plots of log (LLNA EC3) versus log (ATF3 EC15), Log (DNAJB4 EC2), log (GCLM EC2) or log (IL-8 EC4) for sensitizers judged as positive for each marker gene. Of the four marker genes, GCLM EC2 showed a strong correlation with LLNA EC3 (correlation coefficient: R = 0.74, N = 26). Among the 26 sensitizers judged as positive for GCLM, 6 extreme or strong sensitizers showed EC2 values of <0.06, whereas 18 out of the 20 moderate or weak sensitizers showed EC2 values of >0.06 (Table 1 and Fig. 5). On the other hand, IL-8 EC4 and ATF3 EC15 values showed a moderate correlation with LLNA EC3 values (R = 0.59 for IL-8 (N = 41); R = 0.50 for ATF3 (N = 35)). Extreme sensitizers provided lower IL-8 EC4 values (0.006 for OXA, 0.035 for TCSA, 0.034 for 4-nitrobenzylbromide (NBB), and 0.002 for DNCB) and/or ATF3 EC15 values (0.027 for TCSA, 0.05 for NBB and 0.026 for DNCB) than sensitizers with other potency categories (Table 1 and Fig. 5). DNAJB4 EC2 values showed a weak correlation with the LLNA EC3 values (R = 0.37, N = 38), suggesting this value might not contribute to the potency estimation. Based on these results, the following criteria were set to predict skin sensitization potency with the EpiSensA. If the EC value(s) of test chemicals met at least one of following three cases; A) GCLM EC2 ≤ 0.06, B) IL-8 EC4 ≤ 0.01 or C) ATF3 EC15 ≤ 0.04, the test chemicals were judged as extreme or strong sensitizers. If not, the test chemicals judged as positive in the EpiSensA were

Table 3
Comparison of the predictive performance of the existing in vitro tests and that o the EpiSensA.

	EpiSensA	DPRA	KeratinoSensTM	h-CLAT
A. Lipophilic chemicals				
N	29	27	26	26
Sensitivity (%)	93	44	67	46
Specificity (%)	100	100	0	100
Accuracy (%)	93	48	62	50
B. Hydrophilic chemicals				
N	43	43	42	41
Sensitivity (%)	96	81	70	81
Specificity (%)	75	81	93	87
Accuracy (%)	88	81	79	83
C. Pre/pro-haptens				
N	11	11	11	10
Sensitivity (%)	100	55	73	80
Specificity (%)	–	–	–	–
Accuracy (%)	100	55	73	80
D. Overall				
N	72	70	68	67
Sensitivity (%)	94	63	69	64
Specificity (%)	78	83	82	88
Accuracy (%)	90	69	72	70

The number of evaluated chemicals (N) and the predictive performances of the indicated assays for lipophilic (logKow ≥ 3.5), hydrophilic (logKow < 3.5), pre/pro-haptens and the total chemicals are shown based on the results shown in Table 1.

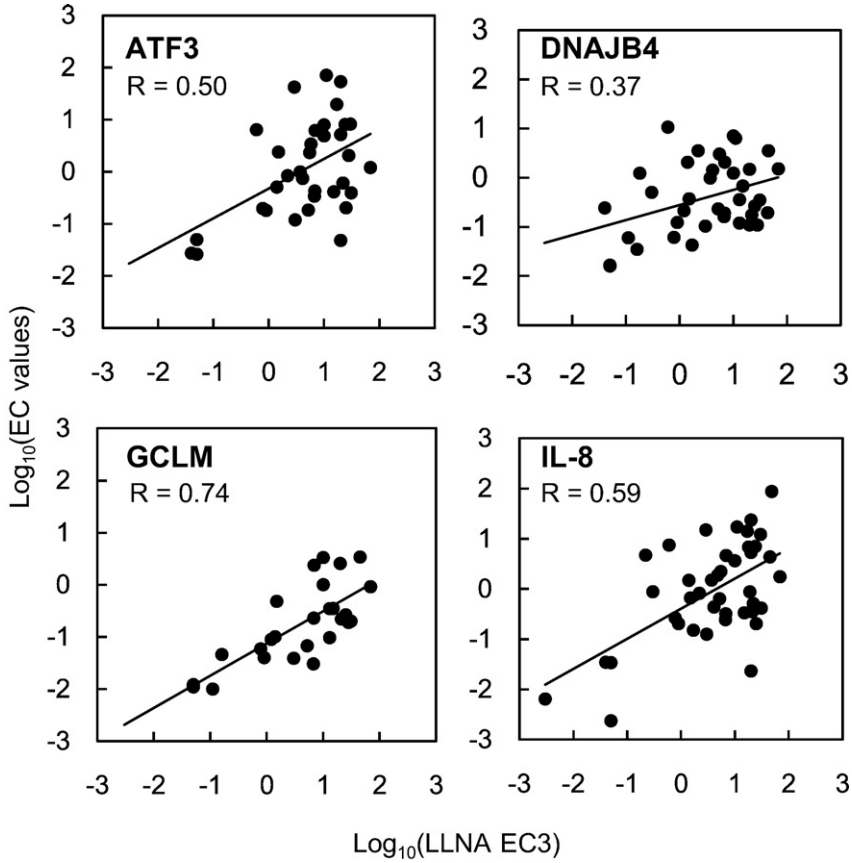

Fig. 5. Scatter plot of the linear fit result of Log (EC value) versus Log (LLNA EC3) for four marker genes. Data of chemicals exceeding the cut-off value of each gene were plotted and the correlation coefficient (R) is shown for each gene. The number of analyzed chemicals was 35 for ATF3, 38 for DNAJB4, 26 for GCLM and 41 for IL-8.

categorized as moderate or weak sensitizers. Table 4 shows the number of test chemicals that were categorized in each potency category of extreme or strong (E or S), moderate or weak (M or W), or non-sensitizer (NS). Eight out of the 12 extreme or strong sensitizers in the LLNA were correctly predicted as extreme or strong in the EpiSensA (Table 4). Also, 37 out of the 42 moderate or weak sensitizers in the LLNA were correctly categorized in the EpiSensA. Consequently, the over prediction rate, under prediction rate and accuracy of the EpiSensA were 8.3%, 9.7% and 82%, respectively (Table 4). These results suggested that EpiSensA would be used to predict potency classification based on the LLNA EC3.

4. Discussion

In this study, we found that the up-regulation of DNAJB4 and GCLM, or ATF3 and IL-8 in NHEKs by a skin sensitizer was partly under the control of Nrf2 or $P2X_7$, which are key molecules for skin sensitization. This finding suggested that the four marker genes of the EpiSensA could be mechanistically relevant for skin sensitization. We also demonstrated that combination of these four marker genes in the RhE model provided excellent predictive performance compared to the LLNA (sensitivity, specificity and accuracy were 94%, 79% and 90%, respectively) for 72 chemicals including 29 lipophilic chemicals and 11-pre/pro-haptens. Finally, we showed that potency classification based on the LLNA EC3 (extreme/strong or moderate/weak) would be predicted based on the threshold concentrations of three marker genes (ATF3, GCLM and IL-8). These data suggested that the EpiSensA, which is based on the expression levels of four marker genes including the new marker IL-8, is a mechanism-based test with good predictive performance of skin sensitization to broad sets of raw materials.

Table 4
Predictive performance of LLNA potency based on the EC values of four marker genes.

		LLNA		
		E or S	M or W	NS
EpiSensA	E or S	8	2	0
	M or W	4	37	4
	NS	0	3	14
Over prediction rate			8.3%	
Under prediction rate			9.7%	
Accuracy			82%	

Prediction of three ranks of sensitization potency: Extreme or Strong (E or S), Moderate or Weak (M or W) or Non-sensitizers (NS), based on the LLNA EC3 values are shown.

4.1. Assessment of the regulation mechanism of EpiSensA marker genes

We showed that Nrf2 was involved in the up-regulation of GCLM and DNAJB4 by sensitizers in NHEKs (Fig. 1). Nrf2 is a key transcription factor that has been reported to negatively regulate the induction of skin sensitization (et al. et al., 2013; van der Veen et al., 2013). Also, multiple Nrf2-dependent genes have been reported to be key markers in discriminating sensitizers from non-sensitizers in RhE models (McKim et al., 2012; Cottrez et al., 2016). Although GCLM has been reported to be an Nrf2 dependent gene (Erickson et al., 2002; MacLeod et al., 2009), there has been no direct evidence that the up-regulation of DNAJB4 in human keratinocytes by a sensitizer is mediated by Nrf2. In this study, we showed for the first time that the induction of DNAJB4 by a skin sensitizer in NHEK cells was regulated through Nrf2. In contrast, Emter et al. reported that the up-regulation of DNAJB4 by DNCB was not regulated through Nrf2 in the human keratinocyte cell line HaCaT that contained a stable insertion of a luciferase gene under the control of the ARE-element of the gene AKR1C2 (Emter et al., 2013). The discrepancy between these results might be due to the difference in the keratinocyte cell used

(reporter cell line vs. primary normal human cell) and/or in the transfection conditions (e.g. reagents, exposure time and concentration of siRNA). The induction level of DNAJB4 by DNCB in the previous study (Emter et al., 2013) was much weaker (<4-fold) compared to our data (10-fold) (Fig. 1), irrespective of whether a similar dose of DNCB was applied. Taking into consideration a previous finding that induction of the DNAJ gene in vivo was Nrf2 dependent (Thimmulappa et al., 2002), it is possible to conclude that Nrf2 was involved, at least in part, in the up-regulation of DNAJB4. The up-regulation of ATF3 by DNCB was augmented by Nrf2 siRNA treatment (Fig. 1B). This result might be due to the secondary effect of Nrf2 knockdown. Nrf2 knockdown reduce the glutathione (GSH) level through the decreased expression of γ-Glutamyl-Cysteinyl-Ligase catalytic subunit (one of the Nrf2 dependent gene) in NHEK (Soeur et al., 2015). It has been reported that GSH depletion in HaCaT increased the generation of reactive oxygen species (ROS) (Inbaraj and Chignell, 2004). Considering the fact that chemical-induced ATF3 expression was meditated through ROS generation (Jeong et al., 2013) and most sensitizers including DNCB have potential to generate ROS (Saito et al., 2013b), it is likely that ROS generation induced by a sensitizer might be involved in the augmented ATF3 expression by Nrf2 knockdown.

On the other hand, the up-regulation of ATF3, as well as of IL-8, was suppressed by KN-62, a specific antagonist of the ATP receptor $P2X_7$ (Fig. 2). Our data showing that the up-regulation of IL-8 was mediated through the $P2X_7$ receptor is consistent with previous reports (Montreekachon et al., 2011; Qiu et al., 2014). On the other hand, our study is the first report to show that the up-regulation of ATF3 by hapten in NHEK cells was mediated through $P2X_7$. Since $P2X_7$ has been reported to be an important positive regulator for the induction of skin sensitization (Weber et al., 2010), our finding suggests that ATF3 might be positively involved in the induction of skin sensitization. This notion is supported by a previous finding by Nguyen et al. (2014) who showed that the production of pro-inflammatory cytokines such as TNF-α, which are involved in the induction of skin sensitization (Kaplan et al., 2012), was impaired in bacteria-infected macrophages of ATF3 KO mice. Our combined data suggested that expression of the four marker genes of ATF3, DNAJB4, GCLM and IL-8 was regulated, at least in part, through the key molecules, Nrf2 and $P2X_7$, of skin sensitization in NHEKs and that these genes could therefore be mechanistically relevant gene markers.

4.2. Comparison between the LabCyte EPI-MODEL and EpiDerm™

In this study, we assessed the predictive performance of four marker genes using the LabCyte EPI-MODEL, which is one of the RhE models adopted in OECD TG439 (OECD, 2013). Our previous study using EpiDerm™ demonstrated that three marker genes (ATF3, DNAJB4 and GCLM) showed good predictive performance for 16 ECVAM reference chemicals (12 sensitizers and 4 non-sensitizers) (Saito et al., 2013a). The expression levels of GCLM genes induced by 16 ECVAM reference chemicals (1.1- to 15.2-fold increase; shown in Table 1) were comparable to those in our previous study (1.0- to 13.8-fold increase) (Saito et al., 2013a). On the other hand, the induction levels of ATF3 and DNAJB4 in the LabCyte EPI-MODEL (1.0- to 77-fold increase in ATF3, 1.0- to 14.7-fold increase in DNAJB4) were slightly lower compared to those in the EpiDerm™ assay (1.5- to 165-fold increase in ATF3; 1.2- to 51-fold increase in DNAJB4). Also, relatively higher induction levels of ATF3 were observed for four reference non-sensitizers (glycerol, lactic acid, salicylic acid and sodium lauryl sulfate), which provided I_{max} values from 1.0- to 12.3-fold in this study (Table 1), compared to the 1.5- to 4.0-fold induction levels observed in our previous study (Saito et al., 2013a). It is unclear why such differences in the expression of some marker genes were observed between the two different RhE models. However, it has been reported that the properties of the stratum corneum barrier (e.g. lamella layer, metabolic enzyme activity) are different between the EpiDerm™ and the Labcyte EPI-MODEL (Kano et al., 2011), which would affect the responsiveness of keratinocytes to topically applied chemicals. Another investigation reported similar findings in which they showed a difference in the production of the inflammatory gene IL-18 between different RhE models (i.e. the EpiDerm™ and the "epiCS® Epidermis Equivalent model", a commercially available RhE model) (Gibbs et al., 2013). Regarding IL-8 induction, McKim et al. (2012) reported that the maximum induction levels of IL-8 induced by the four ECVAM reference skin sensitizers DNCB, 2-mercptbenzothiazole (MBT), isoeugenol and eugenol in the EpiDerm™ were about 10-fold, over 20-fold, 3.5-fold or 2.5-fold, respectively. These values are similar to the I_{max} values of IL-8 obtained in the present study (7.0-fold induction by DNCB, 16-fold by MBT, 2.3-fold by isoeugenol and 2.1-fold by eugenol) (Table 1), suggesting that the induction pattern of IL-8 by sensitizers might be similar in the two RhE models. The combined data suggest that it is possible that the marker genes used in the EpiSensA might be compatible between RhE models, although optimization of the prediction model for each RhE model would be needed to correctly predict skin sensitization.

4.3. IL-8

In this study, we newly added IL-8 as a marker gene for the EpiSensA and showed that most of the tested sensitizers including lipophilic chemicals (i.e. logKow ≥ 3.5) up-regulated the expression of IL-8 (Fig. 3 and Table 1). Although previous findings showed that IL-8 could be a good predictive marker for skin sensitization in monolayer keratinocytes (McKim et al., 2010; Bae et al., 2015), in this paper, we are the first to demonstrate that IL-8 could be a good indicator of skin sensitization for larger sets of chemicals, including lipophilic chemicals, in an RhE model. Weber et al. (2015) recently reported that neutrophils are important immune cells for both the induction and the elicitation of skin sensitization. This finding implies that IL-8, which is a chemokine that mainly promotes the recruitment of neutrophils, could be an important mediator of skin sensitization. On the other hand, IL-8 production can also be observed in other skin inflammations such as in irritant contact dermatitis (Lee et al., 2013), suggesting that IL-8 might be less specific to the induction of skin sensitization. Nevertheless, it is also a fact that in our study five sensitizers (OXA, benzoyl peroxide, benzyl cinnamate, lillial, tridecane) were positive only for induction of IL-8, and three out of these five chemicals were lipophilic chemicals (Table 1), which affected the relatively high sensitivity of IL-8 (Table 2). Thus, our data suggested that IL-8 could be a good marker gene of skin sensitization, especially for decreasing false negatives.

4.4. The advantage of the EpiSensA for the prediction of skin sensitization potential

We showed that the combination of two genes related to inflammatory (ATF3 and IL-8) or cytoprotective (DNAJB4 and GCLM) responses provided better sensitivity and accuracy than a single marker (Table 2). These results suggested that the use of multiple marker genes related to one of two key responses in keratinocytes (inflammatory or cytoprotective) provided better predictivity. Other studies have similarly shown that a combination of multiple genes related to the same signaling pathway (i.e. the Nrf2 pathway) could improve the predictive performance in RhE-based assays (McKim et al., 2012; Cottrez et al., 2016). Finally, we demonstrated that sensitivity and accuracy for 72 chemicals compared to the LLNA were 94% and 90%, respectively by the combination of four marker genes (Table 2), and that these values were relatively higher than those of existing in vitro tests (DPRA, KeratinoSens™ and h-CLAT) (Table 3). This higher predictivity was mainly attributed to a higher capability of detecting lipophilic chemicals (Table 3), which strongly supported our initial idea that the EpiSensA could overcome one of the major limitations of existing in vitro tests, i.e. the inability to assay lipophilic chemicals. Furthermore, the EpiSensA

provided better sensitivity (96%) to hydrophilic chemicals compared to the existing three tests (DPRA, KeratinoSens™ and h-CLAT) (ranging from 70 to 81%, Table 3). The first reason for this higher sensitivity is the improved detection of pre/pro-haptens, whose detection is also a limitation of existing in vitro tests (OECD, 2015a, 2015b and OECD, 2015C). In this study, we demonstrated that EpiSensA could detect all of the 11 tested pre/pro-haptens, which included 6 hydrophilic pre/pro-haptens (i.e. isoeugenol, ethylene diamine, diethylene triamine, resorcinol, cinnamic alcohol and eugenol) that showed false negative results in at least one out of three other tests (Table 1). Although we have not shown that the metabolites of these pre/pro-haptens were produced in our test system, these data suggested that the RhE-based assay EpiSensA, has the capability of predicting pre/pro-haptens. This idea was supported by previous studies, which showed that other RhE based assays could also correctly identify several hydrophilic pre/pro-haptens (McKim et al., 2012; Gibbs et al., 2013; Cottrez et al., 2016). The second reason for the higher sensitivity of the EpiSensA is its ability to detect chemicals whose evaluation is technically difficult using cell-based systems. For instance, benzoyl peroxide, which is a sensitizer that showed a false negative in the cell-based KeratinoSens™ and h-CLAT assays, was correctly predicted in the EpiSensA (dissolved in a lipophilic vehicle, AOO) (Table 1). It has been reported that this chemical would be immediately hydrolyzed to a non-sensitizing chemical, benzoic acid, under aqueous conditions (Aptula et al., 2005). These data indicated that EpiSensA is applicable to chemicals that are unstable in cell-based systems with aqueous media. The third reason for the higher sensitivity of the EpiSensA is that the EpiSensA used four marker genes that reflect two different keratinocyte responses (inflammatory and cytoprotective). EpiSensA is an RhE-based assay, the main constituent of which is keratinocytes. KeratinoSens™ is also a keratinocyte-based assay that is based on Nrf2/ARE activation (cytoprotective response) in the keratinocyte cell-line HaCaT (OECD, 2015b). Some sensitizers such as 3-propylidenephthalide and penicillin G, both of which are acyl transfer agents (Roberts et al., 2007), were positive in the EpiSensA, but were negative in the KeratinoSens™ assay (Table 1). It has been reported that KeratinoSens™ has relatively lower sensitivity to acyl transfer agents because this category of sensitizers cannot activate the Nrf2/ARE pathway (Urbisch et al., 2015). On the other hand, EpiSensA could detect penicillin G by its induction of ATF3 and IL-8 (Table 1), which could be involved in inflammatory pathways (Fig. 2). Also, 3-propylidenephthalide was detected by induction of ATF3, IL-8 and DNAJB4, although the induction level of DNAJB4 was relatively weak (2.3-fold increase). These data indicated that the EpiSensA based on two different keratinocyte responses could provide higher sensitivity than the other keratinocyte-based assays, which focus on only one keratinocyte response. Combining all of these data, it is strongly suggested that the EpiSensA can overcome the limitations of existing in vitro tests in terms of the testing system (RhE-based) and endpoints (four marker genes that relate to two different keratinocyte responses).

Moreover, although some RhE-based skin sensitization assays such as the EE assay and the SENS-IS assay showed >95% accuracy for dozens of chemicals including several pre/pro-haptens, the predictivity of these assays for lipophilic chemicals has not been clearly depicted (Gibbs et al., 2013; Cottrez et al., 2016). Thus, this study is the first report to clearly demonstrate that lipophilic chemicals can be correctly evaluated by a RhE-based skin sensitization assay with high sensitivity (93%). On the other hand, it is also the fact that only two lipophilic non-sensitizers without cytotoxicity were tested in this study. To further assess the predictive performance of the EpiSensA for lipophilic chemicals, we need to test more lipophilic non-sensitizers in the future.

4.5. False negatives and positives

There were three false negatives (tocopherol, squalic acid (SA) and isopropyl myristate (IM)) and four false positives (BKC, MS, OA and SLS) in the EpiSensA, which need to be discussed for better definition of the applicability domain of this assay.

Regarding the three false negatives, it has been reported that tocopherol and IM are essentially free from skin sensitizing activity in humans (Basketter et al., 2014), suggesting the EpiSensA could correctly predict skin sensitization potential in humans and that the LLNA results were probably false positives as suggested by other investigators (Natsch et al., 2013; Urbisch et al., 2015). On the other hand, it has been reported that SA cannot permeate the cell membrane in aqueous vehicles because, at physiological pH it exists as its dianion in aqueous conditions (Aptula et al., 2005). SA was not soluble in AOO (a lipophilic vehicle), but was soluble in 50% EtOH (an aqueous vehicle) at a maximum of 5%, and showed no cytotoxicity in the EpiSensA. These data suggested that there was insufficient exposure of the cells to SA. Similar false outcomes have been observed in other in vitro skin sensitization assays such as the KeratinoSens™ and h-CLAT assays that use an aqueous culture medium (Urbisch et al., 2015). Thus, due care should be taken with interpreting negative outcomes in the EpiSensA when easily ionized chemicals are tested using aqueous vehicles (DW or 50% EtOH in DW).

Regarding false positives, three out of the four false positives (BKC, SLS and MS) induced only IL-8 expression at levels slightly exceeding the cut-off value of 4-fold induction (BKC, 6.2-fold; SLS, 8.6-fold; and MS, 6.0-fold induction). These three chemicals are irritants and the first two are surfactants, suggesting that some irritating chemicals such as surfactants might cause a false positive result in the EpiSensA, by induction of only IL-8 expression. Similar observations have been reported by other investigators using an RhE model. Coquette et al. (2003) reported that SLS, BKC and benzoic acid (an irritant) significantly up-regulated IL-8 mRNA expression in an RhE model, although the induction levels were relatively weak compared to the levels induced by skin sensitizers (e.g. DNCB). Also, it has been reported that SLS weakly induced IL-8 mRNA in the EpiDerm™ assay (McKim et al., 2012). Considering the fact that IL-8 production was observed in irritant contact dermatitis (Lee et al., 2013), relatively weak up-regulation of IL-8 by irritants (surfactants) would be inevitable using RhE models. Thus, when irritating test chemicals such as surfactants up-regulate only IL-8 expression to a level that slightly exceeds the cut-off value (e.g. <10-fold induction), care would need to be taken to judge whether the test chemicals were true positives or not. On the other hand, OA up-regulated the expression of ATF3, DNAJB4 and IL-8 to a level that exceeded the cut-off values (38.1-fold induction of ATF3, 3.5-fold of DNAJB4 and 5.9-fold of IL-8). It remains unclear why this chemical up-regulated three marker genes; however, previous reports showed that OA as well as other fatty acids have the potential to induce ATF3 and DNAJB4 in rat cardiomyocytes (Lockridge et al., 2008), or IL-8 in chicken macrophages (Sunkara et al., 2012), suggesting the fatty acids might have the potential to up-regulate these three marker genes. To further define the applicability domain of the EpiSensA, other middle chain and long chain fatty acids need to be evaluated.

4.6. Potency assessment

In addition to assessment of skin sensitization potential, potency prediction is important for the GHS classification, which goes beyond the hazard identification by defining two classes of sensitizers, weak and strong. For potency prediction, LLNA EC3 values could be useful because of the close correlation with the relative ability of contact allergens to cause skin sensitization in Human (Basketter et al., 2000; Griem et al., 2003). Although human sensitization potency of some fragrance materials (e.g. hexyl salicylate) or nickel might be over- or under-estimated in the LLNA (Urbisch et al., 2015), it has been proposed that LLNA EC3 could be used to estimate human acceptable exposure levels of sensitizers as part of quantitative risk assessment (Loveless et al., 2010). Also, it is important to distinguish between the two potency categories of extreme/strong and moderate/weak, since chemicals

with relatively weak (moderate/weak) skin sensitization potency might be used in a product at very limited concentrations with special care (e.g. based on the sensitization threshold (Keller et al., 2009; Safford et al., 2011)). In this study, we showed that the GCLM EC2 value correlated well with the LLNA EC3 value (Fig. 5). We also demonstrated that the rank categorization of LLNA EC3 values (extreme/strong or moderate/weak) of 72 tested chemicals including 29 lipophilic chemicals and 11 pre/pro-haptens was predicted with good accuracy (82%), based on ATF3 EC15, GCLM EC2 and IL-8 EC4 values (Table 4). Among 12 extreme or strong sensitizers tested in the EpiSensA, hexyl salicylate has been reported to be a very weak human sensitizer with no induction in human tests (Human Repeated Insult Patch Test and Human Maximization Test) at over 20,000 μg/cm^2 (Urbisch et al., 2015). Excluding hexyl salicylate, the predictivity of the EpiSensA for 11 extreme and strong chemicals were 73% (8/11), which was comparable to that of the h-CLAT (74%) for extreme and strong chemicals (Nukada et al., 2012). Thus, these data suggested that the threshold concentrations of EpiSensA marker genes (ATF3, GCLM, IL-8) might be useful to estimate the potency classification based on the LLNA EC3. It has been reported that other RhE-based assays are applicable to the estimation of skin sensitization potency. For instance, Gibbs et al. (2013) showed that the outcome of the EE assay for 11 hydrophilic sensitizers including 5 pre/pro-haptens showed a good correlation with skin sensitization potency in humans and with the LLNA data. Also, Cottrez et al. (2016) reported that the SENS-IS assay provided >90% accuracy for the categorization of 150 tested chemicals into the 5 LLNA potency categories of extreme, strong, moderate, weak and non-sensitizer. In addition to these RhE-based assays, our data showed that the EpiSensA could also provide information regarding the potency classification. On the other hand, it is also a fact that a 1:1 potency assessment (i.e. estimation of the LLNA EC3 values) cannot be made based solely on the result of the EpiSensA. To solve this problem, incorporation of the EpiSensA data into a global ITS system such as the Bayesian network Integrated Testing Strategy (Jaworska et al., 2015), which integrates all of the information (e.g. in vitro data, and physic-chemical properties) for all chemical classes into one model to predict skin sensitization potency might be useful, since the training set of the global ITS system contained limited data of lipophilic chemicals (Natsch et al., 2013). Further study is necessary to precisely predict the skin sensitization potency of broad sets of chemicals.

5. Conclusion and future plans

In summary, the EpiSensA, which is based on the expression levels of four marker genes that are related to keratinocyte responses in the early phase of skin sensitization, is a mechanism-based test with good predictive performance of skin sensitization to broad sets of raw materials. To further examine the utility of EpiSensA as part of a test battery for non-animal skin sensitization assessment, we will expand the dataset to further clarify the applicability domain of the EpiSensA. Also, intra/inter-laboratory reproducibility will be assessed towards establishment of the EpiSensA.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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