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Supplemental Information

Apple Peel and Flesh Contain Pro-neurogenic Compounds

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Supplementary Data

Supplementary Table 1. List of the top 10 upregulated and downregulated genes in quercetin-treated adherent neural precursor cells cultured under differentiation conditions.

Gene ID	Gene symbol	Gene name	Relative expression	Adjusted *p* value
14859	Gsta3	glutathione S-transferase, alpha 3	4.56	0.001
228775	Trib3	tribbles homolog 3 (Drosophila)	3.8	0.002
76650	Srxn1	sulfiredoxin 1 homolog (S. cerevisiae)	3.6	0.001
71839	Osgin1	oxidative stress induced growth inhibitor 1	3.56	0.001
15220	Foxq1	forkhead box Q1	3.38	0.001
23886	Gdf15	growth differentiation factor 15	3.36	0.004
19773	Rln1	relaxin 1	3.2	0.002
14630	Gclm	glutamate-cysteine ligase, modifier subunit	3.14	0.001
53945	Slc40a1	solute carrier family 40 (iron-regulated transporter), member 1	3.1	0.001
12705	Cited1	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	3.07	0.001
64075	Smoc1	SPARC related modular calcium binding 1	-3.81	0.005
16979	Lrrn1	leucine rich repeat protein 1, neuronal	-2.89	0.003
330166	Miat	myocardial infarction associated transcript (non-protein coding)	-2.84	0.002
70433	Draxin	dorsal inhibitory axon guidance protein	-2.78	0.002
18761	Prkcq	protein kinase C, theta	-2.77	0.002
12799	Cnp	2',3'-cyclic nucleotide 3' phosphodiesterase	-2.75	0.004
18377	Omg	oligodendrocyte myelin glycoprotein	-2.68	0.002
70784	Rasl12	RAS-like, family 12	-2.58	0.002
18481	Pak3	p21 protein (Cdc42/Rac)-activated kinase 3	-2.55	0.002
101488	Slco2b1	solute carrier organic anion transporter family, member 2b1	-2.47	0.001

Supplementary Table 2. List of the differential expression of JNKs and related pro-apoptotic genes in the MAPK pathway in quercetin-treated adherent neural precursor cells cultured under differentiation conditions.

Gene ID	Gene symbol	Gene name	Relative expression	Adjusted *p* value
26420	Mapk9	mitogen activated protein kinase-9 (JNK2)	0.49	0.04
26414	Mapk10	mitogen activated protein kinase-10 (JNK3)	0.95	0.01
12028	Bax	BCL2-associated X protein	0.9	0.01
170770	PUMA	BCL2 binding component 3 (Bbc3)	0.53	0.01
12122	Bid	BH3 interacting domain death agonist	1.05	0.002
26419	Mapk8	mitogen activated protein kinase-8 (JNK1)	-0.57	0.02

Supplementary Table 3. A list of the differentially expressed genes mapped to the PI3 kinase-Akt pathway (KEGG pathways mmu04151) in quercetin-treated adherent neural precursor cells cultured under differentiation conditions.

Gene ID	Gene symbol	Gene name	Relative expression	Adjusted *p* value
22339	Vegfa	vascular endothelial growth factor A	2.17	0.002
20393	Sgk1	serum/glucocorticoid regulated kinase 1	1.87	0.002
13685	Eif4ebp1	eukaryotic translation initiation factor 4E binding protein 1	1.48	0.008
14164	Fgf1	fibroblast growth factor 1	1.47	0.005
11911	Atf4	activating transcription factor 4	1.41	0.013
108079	Prkaa2	protein kinase, AMP-activated, alpha 2 catalytic subunit	1.41	0.012
17246	Mdm2	transformed mouse 3T3 cell double minute 2	1.3	0.005
14696	Gnb4	guanine nucleotide binding protein (G protein), beta 4	1.26	0.004
16590	Kit	kit oncogene	1.23	0.016
12048	Bcl2l1	BCL2-like 1	1.11	0.004
12575	Cdkn1	cyclin-dependent kinase inhibitor 1A (P21)	1.02	0.025
241226	Itga8	integrin alpha 8	0.98	0.011
14268	Fn1	fibronectin 1	0.96	0.025
16410	Itgav	integrin alpha V	0.92	0.01
22628	Ywhag	tyrosine 3-monooxygenase/tryptophan 5 monooxygenase activation protein, gamma polypeptide	0.91	0.024
21898	Tlr4	toll-like receptor 4	0.9	0.005
22341	Vegfc	vascular endothelial growth factor C	0.9	0.012

74769	Pik3cb	phosphatidylinositol 3-kinase, catalytic, beta polypeptide	0.88	0.017
12444	Ccnd2	cyclin D2	0.88	0.024
20181	Rxra	retinoid X receptor alpha	0.87	0.004
18750	Prkca	protein kinase C, alpha	0.86	0.01
14083	Ptk2	PTK2 protein tyrosine kinase 2	0.84	0.004
13640	Efna5	ephrin A5	0.84	0.017
208647	Creb3l2	cAMP responsive element binding protein 3-like 2	0.84	0.02
104099	Itga9	integrin alpha 9	0.8	0.017
12443	Ccnd1	cyclin D1	0.77	0.031
192897	Itgb4	integrin beta 4	0.75	0.002
14205	Figf	c-fos induced growth factor	0.75	0.017
26427	Creb3l1	cAMP responsive element binding protein 3-like 1	0.72	0.003
109333	Pkn2	protein kinase N2	0.71	0.002
226849	Ppp2r5a	protein phosphatase 2, regulatory subunit B (B56), alpha isoform	0.7	0.013
16337	Insr	insulin receptor	0.68	0.03
16194	Il6ra	interleukin 6 receptor, alpha	0.65	0.036
15461	Hras	Harvey rat sarcoma virus oncogene 1	0.63	0.036
18607	Pdpk1	3-phosphoinositide dependent protein kinase 1	0.63	0.012
12977	Csf1	colony stimulating factor 1(macrophage)	0.6	0.02
23797	Akt3	thymoma viral proto-oncogene 3	0.6	0.004
14183	Fgfr2	fibroblast growth factor receptor 2	0.56	0.012
12913	Creb3	cAMP responsive element binding protein 3	0.55	0.009
14182	Fgfr1	fibroblast growth factor receptor 1	0.51	0.03
12831	Col5a1	collagen, type V, alpha 1	0.5	0.008
16402	Itga5	integrin alpha 5 (fibronectin receptor alpha)	0.5	0.029

14706	Gng4	guanine nucleotide binding protein (G protein) gamma 4	0.49	0.002
269643	Ppp2r2c	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), gamma isoform	0.48	0.026
16151	Ikbkg	inhibitor of kappaB kinase gamma	0.43	0.015
19053	Ppp2cb	protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	0.43	0.017
19211	Pten	phosphatase and tensin homolog	0.38	0.034
54635	Pdgfc	platelet-derived growth factor, C polypeptide	0.38	0.036
19052	Ppp2ca	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	0.37	0.014
14936	Gys1	glycogen synthase 1, muscle	0.36	0.044
14745	Lpar1	lysophosphatidic acid receptor 1	0.33	0.026
14171	Fgf17	fibroblast growth factor 17	0.3	0.02
14600	Ghr	growth hormone receptor	0.19	0.018
52432	Ppp2r2d	protein phosphatase 2, regulatory subunit B, delta isoform	0.18	0.026
14377	G6pc	glucose-6-phosphatase, catalytic	0.13	0.04
14170	Fgf15	fibroblast growth factor 15	-0.13	0.032
12189	Brca1	breast cancer 1	-0.27	0.016
72508	Rps6kb1	ribosomal protein S6 kinase, polypeptide 1	-0.34	0.041
78134	Lpar4	lysophosphatidic acid receptor 4	-0.35	0.041
22370	Vtn	vitronectin	-0.37	0.006
12445	Ccnd3	cyclin D3	-0.42	0.02
21827	Thbs3	thrombospondin 3	-0.48	0.036
22059	Trp53	transformation related protein 53	-0.50	0.014
16398	Itga2	integrin alpha 2	-0.52	0.031
13684	Eif4e	eukaryotic translation initiation factor 4E	-0.56	0.022

12830	Col4a5	collagen, type IV, alpha 5	-0.66	0.006
12447	Ccne1	cyclin E1	-0.69	0.047
12832	Col5a2	collagen, type V, alpha 2	-0.69	0.012
56716	Mlst8	MTOR associated protein, LST8 homolog (*S. cerevisiae*)	-0.71	0.013
373864	Col27a1	collagen, type XXVII, alpha 1	-0.71	0.029
12125	Bcl2l11	BCL2-like 11 (apoptosis facilitator)	-0.72	0.005
13649	Egfr	epidermal growth factor receptor	-0.72	0.045
17311	Kitl	kit ligand	-0.79	0.025
12566	Cdk2	cyclin-dependent kinase 2	-0.84	0.023
18709	Pik3r2	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2 (p85 beta)	-0.84	0.009
12675	Chuk	conserved helix-loop-helix ubiquitous kinase	-0.9	0.006
21826	Thbs2	thrombospondin 2	-0.95	0.004
12567	Cdk4	cyclin-dependent kinase 4	-0.97	0.005
14168	Fgf13	fibroblast growth factor 13	-1.39	0.014
18595	Pdgfra	platelet derived growth factor receptor, alpha polypeptide	-1.43	0.008
18710	Pik3r3	phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3 (p55)	-1.45	0.003
14708	Gng7	guanine nucleotide binding protein (G protein), gamma 7	-1.49	0.002

Supplementary Table 4. A list of the differentially expressed genes mapped to the Nrf2-Keap1 pathway (Wikipathways WP1245) in quercetin-treated adherent neural precursor cells cultured under differentiation conditions.

Gene ID	Gene symbol	Gene name	Relative expression	Adjusted *p* value
14859	Gsta3	glutathione S-transferase, alpha 3	4.56	0.001
12608	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	1.96	0.004
15368	Hmox1	heme oxygenase (decycling) 1	2.24	0.003
17132	Maf	avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog	0.36	0.033
18104	Nqo1	NAD(P)H dehydrogenase, quinone 1	1.91	0.002
18750	Prkca	protein kinase C, alpha	0.86	0.01
14630	Gclm	glutamate-cysteine ligase, modifier subunit	3.14	0.001
50868	Keap1	kelch-like ECH-associated protein 1	0.53	0.01
14629	Gclc	glutamate-cysteine ligase, catalytic subunit	0.87	0.006
18024	Nfe2l2	nuclear factor, erythroid derived 2, like 2	0.34	0.03
26419	Mapk8	mitogen-activated protein kinase 8	-0.57	0.021

Supplementary Table 5. The total flavonoid content in different apple varieties.

Cultivar	Total flavonoid content (equivalent to mg quercetin / 100 g fresh weight)	
	Peel	**Flesh**
Pinova	379 ± 93.95	42.45 ± 17.63
Elstar	428.9 ± 103.7	96.74 ± 32.61
Pilot	344.3 ± 74.19	67.36 ± 25.43
Rebella	186.6 ± 44.4	48.82 ± 15.10
Roter Berlepsch	148.1 ± 60.55	18.74 ± 7.86
Jonagold	87.85 ± 28.45	36.98 ± 15.53

Supplementary Table 6. Peak annotation of UPLC / PDA / ESI-QTOFMS analysis of fraction IV based on accurate mass measurement and high-resolution CID mass spectra.

Peak number	*m/z*, ESI($^+$)	*m/z*, ESI($^-$)	Elemental composition	Comments
1	347.0942, $[M^+Na]^+$	323.0979, $[M^-H]^-$	$C_{12}H_{20}O_{10}$	hexoside
2	339.0668, $[M^+Na]^+$	315.0713, $[M^-H]^-$	$C_{13}H_{16}O_9$	dihydroxybenzoate O-hexoside
3	361.1099, $[M^+Na]^+$	337.1133, $[M^-H]^-$	$C_{13}H_{22}O_{10}$	hexoside, homolog of 1
4	471.1082, $[M^+Na]^+$	447.1132, $[M^-H]^-$	$C_{18}H_{24}O_{13}$	dihydroxybenzoate *O*-(hexoside-pentoside)
5	361.1091, $[M^+Na]^+$	337.1140, $[M^-H]^-$	$C_{13}H_{22}O_{10}$	hexoside, Isomer of 3
6	660.1686, $[M^+H]^+$	658.1546, $[M^-H]^-$	?	?

Supplementary Table 7. List of antibodies

Antibody	Dilution	Supplier	Catalogue #
Primary antibodies			
Goat anti Doublecortin	1:250	Santa Cruz Biotechnology	sc-8067
Mouse anti Idu/BrdU	1:500	BD Bioscience	347580
Mouse anti Map2(ab)	1:1000	Sigma	M-1406
Mouse anti Nestin	1:400	BD Bioscience	611658
Mouse anti S100β	1:1000	Sigma	S 2532
Mouse anti β-Actin	1:1000	Santa Cruz Biotechnology	sc-81178
Mouse anti β-III-Tubulin	1:1000	Promega	G712A
Rabbit anti Vinculin	1:1000	ThermoFisher Scientific	700062
Rabbit anti Cdk4	1:1000	Abcam	ab7955
Rabbit anti HCAR-1	1:100	Sigma	SAB1300090
Rabbit anti cleaved Caspase-3	1:1000	Cell Signaling Technology	9661
Rabbit anti Cyclin D1	1:1000	Abcam	ab16663
Rabbit anti FOX3/NeuN	1:500	Abcam	ab104225
Rabbit anti GFAP	1:2000	Dako	Z0334
Rabbit anti GSTA3	1:1000	Proteintech	16703-1-AP
Rabbit anti Ki67p (NCL)	1:500	Novocastra	NCL-Ki67p
Rabbit anti p27 (C-19)	1:1000	Santa Cruz Biotechnology	sc-528
Rabbit anti phospho-Akt	1:2000	Cell Signaling Technology	4060S
Rabbit anti SOX2	1:500	Merck Millipore	AB5603
Rabbit anti-BCL2L1	1:400	Sigma	AV30475
Rat anti-BrdU	1:500	AbD Serotec	OBT0030
Secondary antibodies			
Donkey anti goat Biotin	1:500	Jackson ImmunoResearch	705-065-147
Donkey anti goat HRP	1:500	Jackson ImmunoResearch	705-035-003
Donkey anti mouse Cy3	1:500	Jackson ImmunoResearch	715-505-151
Donkey anti mouse Cy5	1:500	Jackson ImmunoResearch	715-175-151
Donkey anti mouse DyLight 488	1:500	Jackson ImmunoResearch	715-485-150
Donkey anti rabbit Biotin	1:500	Jackson ImmunoResearch	711-065-152
Donkey anti rabbit Cy3	1:500	Jackson ImmunoResearch	711-165-152
Donkey anti rat Biotin	1:500	Jackson ImmunoResearch	712-065-153
Donkey anti rat Cy 3	1:500	Jackson ImmunoResearch	712-225-153
Goat anti mouse HRP	1:500	Jackson ImmunoResearch	115-035-003
Goat anti rabbit horseradish peroxidase (HRP)	1:500	Jackson ImmunoResearch	111-035-003
Goat anti rat HRP	1:500	Jackson ImmunoResearch	112-035-003

Supplementary Table 8. Primer sequences

Primer name	Sequence (5'-3')
Bcl2l1 Forward	GCGTGGAAAGCGTAGACAAG
Bcl2l1 Reverse	GCTGCATTGTTCCCGTAGAG
Bcl2l11 Forward	GCCAGGCCTTCAACCACTAT
Bcl2l11 Reverse	GCTCCTGTGCAATCCGTATC
β-actin Forward	TGACCCAGATCATGTTTGAGA
β-actin Reverse	GGAGAGCATAGCCCTCGTA

Supplemental Experimental Procedures

Apple fractionation

The first fractionation steps were performed using the solvent fractionation method. The extracts were dissolved in ddH_2O and 1 volume of ethyl acetate was added. Using a separation funnel, the water and ethyl acetate phase was separated and collected. The solvent was evaporated, and the water fraction was further separated in the mixture of water and butanol (1:1). From these steps, 3 fractions were obtained each from apple peel and the solvent in all fractions was evaporated until maximum dryness. The active water fraction from the butanol separation was further fractionated by column separation using a Reverse phase C8 column (Vydac). The fraction was dissolved in 20% methanol solution before being injected into the column and then eluted with 20%-, 40%-, 60%- and 100% methanol consecutively for 30 minutes each. The elutes were then dried using centrifugal evaporator prior to being tested in the neurosphere assay. The active fraction (the one collected in 40% methanol) was further fractionated using Eurosphere C18 column using a gradient system with 1% aqueous acetic acid and 100 % acetonitrile (ACN) as the solvent. The gradient started with 80% ACN, then after 20 minutes decreased to 50% ACN, further decreased to 30% ACN within 20 minutes and finally within 20 minutes to 5%, followed by an equilibration step to the initial run condition. In this separation technique, 4 fractions were collected from different time ranges, i.e. minute 0-10, 10-20, 20-30 and 30-60, respectively. The solvent was evaporated in centrifugal evaporator prior to the neurosphere assay. The active fraction from this step was then analyzed using UPLC-MS system for structure elucidation.

Compound elucidation by UPLC/ESI-QTOFMS

Chromatographic separations were performed at 40°C on an Acquity UPLC system (Waters) equipped with a HSS T3 column (100 Å~ 1.0 mm, particle size 1.8 µm, Waters) applying the

following binary gradient at a flow rate of 150 µl min-1: 0-1 min, isocratic 95% A (water/formic acid, 99.9/0.1 v/v), 5% B (acetonitrile/formic acid, 99.9/0.1 v/v); 1-16 min, linear from 5 to 50% B; 16-18 min, isocratic 95% B; 18-20 min, isocratic 5% B. The injection volume was 2.6 µl (full loop injection). Eluting compounds were detected from 190-500 nm using a photodiode array detector (Waters) and from m/z 100-1000 using a MicrOTOF-Q hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an Apollo II electrospray ion source in negative ion mode. The following instrument settings were applied: nebulizer gas, nitrogen, 1.6 bar; dry gas, nitrogen, 6 l/min, 190°C; capillary, +4000 V; end plate offset, -500 V; funnel 1 RF, 200 V; funnel 2 RF, 200 V; in-source CID energy, 0 V; hexapole RF, 100 V; quadrupole ion energy, 3 eV; collision gas, argon; collision energy, 10 eV; collision RF 200/400 V (timing 50/50); transfer time, 70 µs; pre pulse storage, 5 µs; pulsar frequency, 10 kHz; spectra rate, 3Hz. Mass spectra were acquired in centroid mode. Calibration of m/z was performed for individual raw data files on lithium formate cluster ions obtained by automatic infusion of 20 µl lithium hydroxide (10 mM) in the mixture of isopropanol/water/formic acid, 49.9/49.9/0.2 (v/v/v) respectively at the end of the gradient using a diverter valve. For the acquisition of collision-induced dissociation mass spectra, appropriate precursor ions were isolated using the first quadrupole (isolation width m/z 4) and fragmented inside the collision cell applying collision energies in the range of 15-30eV.

Total flavonoid assay

Total flavonoids in apple cultivars were measured using the sodium borohydride/chloranil-based assay as described previously (He and Liu, 2008). Dried samples and quercetin standards were reconstituted in 1 ml THF/EtOH (1:1,v/v) in a glass test tube. 0.5 ml of 50 mM NaBH4 and 0.5 ml of 74.56 mM AlCl3 solution, were added to each tube followed by a 30 min mixing step at RT. An additional 0.5 ml of NaBH4 solution was added with continuing shaking for a further 30 min at

RT. Subsequently, 2 ml of 0.8 M of cold acetic acid solution was added, thoroughly mixed and then incubated in the dark for 15 min. The tubes were then heated to 100ºC with shaking for 60 min after adding 1ml of 20 mM chloranil. The tubes were cooled and the final volume was brought to 4 ml with methanol. Then, 1 ml of 1052 mM vanillin was added into each tube and mixed thoroughly. Finally, 2 ml of concentrated HCl was added into each tube and kept in the dark for 15 min after a thorough mix. For detection, 200 µl of final reaction solution was added into each well of a 96-well plate and the absorbances were measured using a microplate reader at 490 nm.

Immunostaining

In vitro immunostaining

The fixed cells were washed twice with 0.9% NaCl and then incubated in 1N HCl at 37°C for 30 min, followed by one wash with 0.1M borate buffer, pH 8, and two washes with PBS. The blocking step was performed in 10% donkey serum and 0.2% Triton X-100 in PBS at RT for 1 h. Anti-BrdU antibody (1:500) in antibody solution (3 % donkey serum and 0.1 % Triton X-100) was subsequently dispensed on each coverslip and incubated either for 2 h at RT or overnight at 4°C. After three washes with PBS, the cells were incubated with fluorescence secondary antibody (1:500, in 3 % donkey serum and 0.1 % Triton X-100) for 90 min at RT. Following two washes with PBS cells were incubated for 10 min with Hoechst 33342 (1:3000 in PBS). After a final two washes with PBS, the coverslips were mounted on glass slides using Aqua-Poly/Mount for fluorescence microscopy. Cell counting was performed using a Leica DM5500B fluorescence microscope with StereoInvestigator software. The number of $BrdU^+$ cells in a given population of cells was quantified from several visual fields and the average was presented as the cell percentage. For all other fluorescence staining the above protocol was followed but omitting the HCl step. For HCAR-1 staining, 0.2% Triton X-100 was replaced with 0.2 % saponin.

Fluorescence and DAB immunostaining on brain tissue

A one-in-six series of sections was blocked in blocking solution for 1 h at RT prior to overnight incubation with primary antibodies at 4ºC. After a washing step with PBS, incubation with fluorescence secondary antibody was done for 3-4 h at RT. The tissues were subsequently mounted on glass coverslips with Aqua Polymount. For 3,3'-diaminobenzidine (DAB) staining, the sections were initially incubated with 0.6% H_2O_2 for 30 min. After washing with TBS, sections were incubated with 1 h blocking solution followed by overnight incubation with primary antibodies. The sections were incubated with biotinylated secondary antibodies for 4 h at RT followed by incubation with avidin/biotinylated enzyme complex solution (ABC elite) for 1 h at RT. Thorough washing steps were performed between incubation steps. The chromogen DAB was used as the enzyme substrate. The sections were mounted on gelatin-coated glass slide, dehydrated in Neoclear® solution and then covered with a glass coverslip in Neo-mount® solution. Images were acquired using a Plan-Apochromat 20x/0.8 air objective on the LSM 780.

Cell quantification in brain sections (BrdU-, CldU-, DCX- or IdU-labeled cells)

The number of labeled cells was determined in series of every sixth section. Positive cells were counted throughout the rostro-caudal axis of the granule cell layer using 40x objectives. The resulting numbers were multiplied by six to estimate the total number of labeled cells. For phenotyping, the level of colocalization between $NeuN^+$ or $S100\beta^+$ cells was determined in 100 randomly selected cells for each dentate gyrus and displayed as a percentage.