Identification of Proteins Interacting with PCSK9 Using a Protoarray Human Protein Microarray

Catherine J. Wooten[a,#], Sreevidhya T. Krishnaji[b,#], Quantil M. Melendez[a], Dayami Lopez[a,*]

[a]Department of Pharmaceutical Sciences, Biomanufacturing Research Institute and Technology Enterprise (BRITE), College of Arts and Sciences, North Carolina Central University, Durham, NC 27707, USA

[b]INSPIRE Faculty, Department of Chemistry, Indian Institute of Science Education and Research, Madhya Pradesh, Bhopal 462066 India

Abstract

Proprotein convertase subtilisin-kexin 9 (PCSK9) appears to be involved in multiple processes. A ProtoArray Human Protein Microarray was used to identify proteins interacting with biotinylated PCSK9. Fifteen novel proteins interacting with PCSK9 were identified using this technique. Only two of these proteins, sterol carrier protein 2 and hepatoma-derived growth factor, related protein 3, have known functions. The identification of proteins that could affect the expression/function of PCSK9 is of great interest due to potential implications in personalized medicine for hypercholesterolemic patients.

Keywords

Protoarray; Protein-protein interaction; Hypercholesterolemia; PCSK9; Metabolic diseases

Introduction

Hypercholesterolemia, which is a major issue in the United States due to the number of patients that suffer this disease, is well-known to improve with diet, exercise, and in the majority of the case, with the use of anti-hypercholesterolemic treatments [1]. Unfortunately, some hypercholesterolemic patients cannot achieve their recommended target levels for low density lipoprotein (LDL)-cholesterol usually due to resistance to statin [2,3]. Interestingly, a gene that has been identified as responsible for statin resistance is proprotein convertase subtilisin/kexin 9 (PCSK9) [4]. Another group of patients (10–25%) suffer from statin intolerance limiting the use of these drugs in these patients [2,3]. Other side effects reported for statins are diabetes [5,6], reduced mental performance [7], and cataract development [8]. However, these adverse effects usually happen in a small number of patients. PCSK9 is the

*Corresponding author: Dr. Dayami Lopez, Department of Pharmaceutical Sciences, BRITE, College of Arts and Sciences, North Carolina Central University, Durham, NC 27707, USA; lopezd@nccu.edu.
#Contributed equally to manuscript

third gene identified in causing familial hypercholesterolemia (FH) in an autosomal dominant manner [9,10]. Serum levels of PCSK9 are not only associated positively with LDL levels in FH patients but also appear to contribute to the phenotypic severity of the FH disorder [11,12]. Both gain-of-function (GOF) and loss-of-function (LOF) mutations in the PCSK9 gene have been reported. The GOF mutations of PCSK9 cause hypercholesterolemia and a higher risk of atherosclerotic-related diseases [13,14]. The LOF mutations, on the other hand, result in hypocholesterolemia and protection against cardiovascular diseases [15,16].

The majority of the PCSK9 present in the serum comes from the liver, small intestines, and kidneys [17]. This convertase controls serum LDL levels by controlling the expression of the LDL receptor at the plasma membrane, especially in hepatic cells, by promoting receptor degradation in the lysosome [18,19]. PCSK9 also degrades the very low-density lipoprotein (VLDL) receptor, the apolipoprotein (Apo) E receptors 1 and 2 (ApoER and ApoER2), the cluster of differentiation 36 and 81, beta-secretase 1, and the epithelial (NA+) channel [20–23]. High PCSK9 levels appear to lead to or are the consequence of, the accumulation of Apo B-containing lipoproteins in the circulation, obesity, diabetes, inflammation, atherosclerotic plaque development, thrombosis, hypertension, and apoptosis (reviewed in [24]). Also, PCSK9 is upregulated during cerebral ischemia, myocardial infarction, kidney disease, and hepatic cancer [24]. Having PCSK9 is also beneficial since it is involved in brain development, especially the cerebellum, liver regeneration, and prevention of infections [24]. To fully comprehend how PCSK9 can control multiple pathways, we need to continue finding protein partners of PCSK9. The objective of this study was to perform ProtoArray to identify proteins that interact with biotinylated-PCSK9 in vitro. The identification of proteins that may influence the expression/function of PCSK9 could assist in the development of personalized treatment options for hypercholesterolemic patients.

Materials and Methods

Biotinylation of rPCSK9 –

Before performing ProtoArray microarrays, recombinant (r) PCSK9 (carrier-free; R&D Systems; Minneapolis, MN) was biotinylated using the EZ Link Sulfo-NHS-SS Biotinylation kit (Pierce Thermo Scientific; Rockford, IL). First, to exchange buffers, 10 μg of rPCSK9 was added to a Zeba™ spin desalting column that has been pre-equilibrated according to the manufacturer's protocol. Centrifugation was performed at $1000 \times g$ for 2 minutes, and the resulting flow-through that contained the rPCSK9 protein in PBS was used in the next step. Sulfo-NHS-SS-Biotin was prepared by dissolving in ultrapure water at the concentration of 10 mM. Biotinylation of rPCSK9 was done with an at least 40-fold molar excess of Sulfo-NHS-SS-Biotin for 1 hour at room temperature. Filtering through another pre-equilibrated Zeba™ spin desalting column was performed as described above to remove excess biotin. Alternatively, excess biotin was removed by filtering through a 3 kDa Amicon ultra-2 centrifugal unit (Fisher Scientific; Pittsburg, PA). Protein concentrations were measured using a NanoDrop 2000 (Fisher Scientific). A HABA assay (reagents provided with the Biotinylation kit) was used to measure biotinylation levels. Calculations for the HABA assay were performed according to the protocol provided with the assay.

Protein Electrophoresis –

Equivalent amounts of biotinylated protein samples were denatured in NuPAGE® LDS Sample buffer (Invitrogen ThermoFisher Scientific; Carlsbad, CA) supplemented with 20 mM DTT at 70 oC for 5 minutes. These proteins were then subjected to electrophoresis on precast NuPAGE™ 4–12% Bis-Tris protein gels (Invitrogen) and NuPAGE® MES SDS Running Buffer (Invitrogen). Electroblotting onto nitrocellulose membranes using NuPAGE® Transfer Buffer (Invitrogen) and staining with 0.1% Ponceau S (in 5% acetic acid; Fisher Scientific) to protein loading and transfer were performed using standard methods [25].

Western Blotting Analysis –

This technique was performed mostly as previously described [25]. Blocking with 2% BSA-TBS was performed for 30 minutes at room temperature. Incubation with the primary antibody mouse anti-PCSK9 (Cayman Chemicals; Ann Arbor, MI; diluted 1:1000) was performed overnight at 4°C. After incubating with the HRP-labeled anti-mouse secondary antibody (Fisher Scientific; diluted 1:2,000) or avidin-HRP (Fisher Scientific; diluted 1:500) for 1 hour at room temperature, the SuperSignal West Pico Chemiluminescence Substrate (Pierce ThermoFisher) was used for detection. Several exposures ranging from 0.5 s to 30 minutes were made using a Kodak Image Station 4000R Pro Imaging System (Bend, OR) and the Carestream Molecular Imaging Software-Standard Edition-v.5.4.2.18893 (New Haven, CT).

Probing of the ProtoArray for Protein-Protein Interaction –

The blocking (50 mM HEPES pH 7.5, 200 mM NaCl, 0.08% Triton X-100, 25% glycerol, 20 mM reduced glutathione, 1X synthetic block, 1 mM DTT) and washing (1X PBS, 0.1% Tween 20, 1X synthetic block) buffers were prepared according to the manufacturer's instructions. First, the ProtoArray (PAH0525011; Invitrogen) was set at 4°C for 15 minutes to equilibrate to the new temperature. The array was then placed with barcode facing up into a well of a pre-chilled chambered incubation tray. Blocking buffer was added followed by incubation for 1 hour at 4°C with shaking. While waiting, the biotinylated rPCSK9 protein was diluted to a final concentration between 5 – 50 μg/mL in a 120 μl volume. After discarding the Blocking buffer and briefly placing the array on a paper towel to remove excess buffer, the array was put back into the tray, and the diluted biotinylated rPCSK9 was added directly onto the array starting at the top. Carefully, a LifterSlip (Invitrogen) was placed onto the slide, letting the probe disperse over the array. The array was then incubated for 90 min at 4°C, trying to keep it flat and facing up with no shaking. Cold wash buffer was added after the incubation followed by removal of the LifterSlip using forceps. Washing was performed for 5 min at 4°C with shaking. The washing buffer was removed; washing was repeated for a total of five times. Streptavidin-Dylight 650 conjugate (Pierce ThermoScientific) was prepared by diluting to 1 μg/mL in 5 mL wash buffer. After the last wash, the diluted Streptavidin-Dylight 650 was added to the tray. Incubation was done at 4°C with shaking for 90 minutes. Washing was repeated as before for a total of five times. After the last wash, the array was removed from the tray with forceps and dipped into a 50-mL conical tube filled with room temperature distilled water. The array was dried by

centrifuging at $200 \times$ g for 2 min in a 50-mL conical tube covered in foil to protect from light. The array was then placed facing down on a LiCor Odyssey scanner and scanned with the following settings: 337 μm resolution, high quality, 0.0 mm focus offset, 700 channels. The output image was saved as a 16-bit TIFF file. Data from three independent arrays were analyzed using the ProtoArray Prospector Imager/Analyzer software 5.2.3 (Invitrogen). The GAL files corresponding to the specific arrays were downloaded from ProtoArray Central (Invitrogen.com) and used to define the array grids. About 9375 proteins, in duplicate, were included in each array.

Results and Discussion

Human rPCSK9 was first biotinylated as indicated in Materials and Methods. Figure 1 shows the results for rPCSK9 biotinylated using 40 molar excess of biotin followed by purification with Zeba™ spin columns. Other samples were loaded onto the gel for comparison. As shown, only the samples containing biotinylated PCSK9 had detectable levels of both PCSK9 (detected using the PCSK9 specific antibody) and biotin (detected using avidin-HRP). Samples containing unbiotinylated PCSK9 (aliquots from PCSK9 exchanged with PBS and the original PCSK9 stock) were only detected with the PCSK9 antibody. Other molar excess concentrations of biotin and alternative methods to remove unreactive biotin molecules were tested as well. Using 200 molar excess of biotin followed by purification with the spin columns (recommended purification method) provided good biotinylation levels that were confirmed as described in Figure 1 and using the HABA assay; each molecule of rPCSK9 was labeled with 15.04 molecules of biotin (data not shown). Using filtration to remove unreactive biotin appeared to result in a rPCSK9 sample with the highest level of biotinylation (226 molecules of biotin/molecule of rPCSK9). However, due to the low detection of PCSK9 in the filtered sample using Western blotting, it was decided to perform the ProtoArray with biotinylated PCSK9 samples purified using the recommended method.

Three independent ProtoArray human protein microarrays were probed with biotinylated rPCSK9. Figure 2 illustrates a typical whole slide image as acquired using a LiCor Odyssey scanner. The images were then aligned to grips provided by the GAL files specific for that array (Invitrogen) using ProtoArray Prospector Imager. The data generated were analyzed in the ProtoArray Prospector Analyzer. Table 1 shows the proteins that interacted with biotinylated rPCSK9 in all three ProtoArray microarrays.

None of the proteins in Table 1 have been reported previously as proteins interacting with PCSK9. Furthermore, only the functions of two of these proteins, sterol carrier protein 2 (SCP2) and hepatoma-derived growth factor, related protein 3 (HDGFRP3), have been reported [26,27]. SCP2 is involved in the transport of lipids and cholesterol between different sides of the cellular membrane and is highly expressed in the liver [26,28]. It has been demonstrated that SCP2 levels are significantly reduced in the liver during diabetes, in association with a significant rise in serum cholesterol levels [29]. Thus, it might be possible that PCSK9 works together with SCP2 during diabetes causing deleterious effects that may worsen the disease. The function of HDGFRP3 is less known, but it has been implicated in cell proliferation [29]. The highest expression of HDGFRP3 has been located in testes and

brain [27]. Other proteins that interacted with biotinylated PCSK9 in at least one array were adrenomedullin (2), macrophage migration inhibitory factor (2), alcohol dehydrogenase (2), glyceraldehyde-3-phosphate dehydrogenase (2), CYP4A11 (2), caveolin-3 (2), protein phosphatase 2 (1), TNF receptor-associated factor 6 (1), presenilin enhancer 2 homolog (1), thyroid hormone receptor interactor 6 (1), fibronectin-1 (1), glycogen synthase kinase 3 beta (1), platelet-derived growth factor receptor-β polypeptide (1), SERPINF1 (1), and SERPINA3 (1). The number within the parenthesis refers to the number of arrays in which interaction was detected.

Learning more about the different functions of PCSK9 and which proteins can modulate the function of this convertase is critical. Many hypercholesterolemic patients can utilize statins, but those that cannot, will need to rely on PCSK9 inhibitors [30,31]. The main problem with these inhibitors is their cost and the possibility of developing severe side effects [30,31]. The proteins identified herein require further confirmation of their interaction in vivo with PCSK9 and whether they can modify PCSK9's function. However, they provide a starting point for the identification of novel therapeutic targets to develop personalized treatment options for hypercholesterolemic patients.

Acknowledgments

References

1. Huijgen R, Vissers MN, Defesche JC, et al. Familial hypercholesterolemia: current treatment and advances in management, Expert Rev Cardiovasc Ther 2008; 6: 567–581. [PubMed: 18402545]

2. Pedersen TR. The Success Story of LDL Cholesterol Lowering. Circ Res 2016; 118: 721–731. [PubMed: 26892969]

3. Guyton JR, Bays HE, Grundy SM, et al. An assessment by the Statin Intolerance Panel: 2014 update, J Clin Lipidol 2014; 8: S72–81. [PubMed: 24793444]

4. Reiner Z. Resistance and intolerance to statins, Nutrition, metabolism, and cardiovascular diseases: NMCD 2014; 24: 1057–1066.

5. Maki KC, Ridker PM, Brown WV, et al. An assessment by the Statin Diabetes Safety Task Force: 2014 update. J Clin Lipidol 2014; 8: S17–29. [PubMed: 24793439]

6. Carter AA, Gomes T, Camacho X, et al. Risk of incident diabetes among patients treated with statins: population-based study, BMJ 2013; 346: f2610.

7. King DS, Wilburn AJ, Wofford MR, et al. Cognitive impairment associated with atorvastatin and simvastatin. Pharmacotherapy 2003; 23: 1663–1667. [PubMed: 14695047]

8. Leuschen J, Mortensen EM, Frei CR, Mansi EA, Panday V., Mansi I., Association of statin use with cataracts: a propensity score-matched analysis. JAMA Ophthalmol 2013; 131: 1427–1434. [PubMed: 24052188]

9. Gu HM, Zhang DW. Hypercholesterolemia, low density lipoprotein receptor and proprotein convertase subtilisin/kexin-type 9. J Biomed Res 2015; 29: 356–361. [PubMed: 26445568]

10. Di Taranto MD, D'Agostino MN, Fortunato G. Functional characterization of mutant genes associated with autosomal dominant familial hypercholesterolemia: integration and evolution of genetic diagnosis, Nutrition, metabolism, and cardiovascular diseases: NMCD 2015; 25: 979–987.

11. Huijgen R, Fouchier SW, Denoun M, et al. Plasma levels of PCSK9 and phenotypic variability in familial hypercholesterolemia, J lipid Res 2012; 53: 979–983. [PubMed: 22375030]

12. Lambert G, Petrides F, Chatelais M, et al. Elevated plasma PCSK9 level is equally detrimental for patients with nonfamilial hypercholesterolemia and heterozygous familial hypercholesterolemia, irrespective of low-density lipoprotein receptor defects. J Am Coll Cardiol 2014; 63: 2365–2373. [PubMed: 24632287]

13. Adi D, Xie X, Liu F, et al. Relationships between genetic polymorphisms of E670G in PCSK9 gene and coronary artery disease: a meta-analysis. Int J Clin Exp Med 2015; 8: 13251–13258. [PubMed: 26550250]

14. Drouin-Chartier JP, Tremblay AJ, Hogue JC, et al. The contribution of PCSK9 levels to the phenotypic severity of familial hypercholesterolemia is independent of LDL receptor genotype. Metabolism 2015; 64: 1541–1547. [PubMed: 26371983]

15. Hooper AJ, Marais AD, Tanyanyiwa DM, et al. The C679X mutation in PCSK9 is present and lowers blood cholesterol in a Southern African population. Atherosclerosis 2007; 193: 445–448. [PubMed: 16989838]

16. Sirois F, Gbeha E, Sanni A, et al. Ethnic differences in the frequency of the cardioprotective C679X PCSK9 mutation in a West African population. Genet Test 2008; 12: 377–380. [PubMed: 18652535]

17. Langhi C, Le May C, Gmyr V, et al. PCSK9 is expressed in pancreatic delta-cells and does not alter insulin secretion. Biochem Biophys Res Commun 2009; 390: 1288–1293. [PubMed: 19878649]

18. Cameron J, Holla OL, Ranheim T, et al. Effect of mutations in the PCSK9 gene on the cell surface LDL receptors. Hum Mol Genet 2006; 15: 1551–1558. [PubMed: 16571601]

19. Saavedra YG, Day R, Seidah NG. The M2 module of the Cys-His-rich domain (CHRD) of PCSK9 protein is needed for the extracellular low-density lipoprotein receptor (LDLR) degradation pathway. J Biological Chem 2012; 287: 43492–43501.

20. Poirier S, Mayer G, Benjannet S, et al. The proprotein convertase PCSK9 induces the degradation of low-density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2. J Biological Chem 2008; 283: 2363–2372.

21. Demers A, Samami S, Lauzier B,C, et al. PCSK9 Induces CD36 Degradation and Affects Long-Chain Fatty Acid Uptake and Triglyceride Metabolism in Adipocytes and in Mouse Liver. Arterioscler Thromb Vasc Biol 2015; 35: 2517–2525. [PubMed: 26494228]

22. Jonas MC, Costantini C, Puglielli L. PCSK9 is required for the disposal of non-acetylated intermediates of the nascent membrane protein BACE1. EMBO Reports 2008; 9: 916–922. [PubMed: 18660751]

23. Le QT, Blanchet M, Seidah NG, et al. Plasma Membrane Tetraspanin CD81 Complexes with Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) and Low-Density Lipoprotein Receptor (LDLR), and Its Levels Are Reduced by PCSK9. J Biological Chem 2015; 290: 23385–23400.

24. Melendez QM, Krishnaji ST, Wooten CJ, et al. Hypercholesterolemia: The role of PCSK9. Arch Biochem Biophys 2017; 625–626: 39–53.

25. Wooten CJ, Adcock AF, Agina-Obu DI, et al. Having excess levels of PCSK9 is not sufficient to induce complex formation between PCSK9 and the LDL receptor. Arch Biochem Biophys 2014; 545: 124–132. [PubMed: 24486405]

26. Szyperski T, Scheek S, Johansson J, et al. NMR determination of the secondary structure and the three-dimensional polypeptide backbone fold of the human sterol carrier protein 2. FEBS Lett 1993; 335: 18–26. [PubMed: 8243660]

27. Ikegame K, Yamamoto M, Kishima Y, et al. A new member of a hepatoma-derived growth factor gene family can translocate to the nucleus. Biochem Biophys Res Commun 1999; 266: 81–87. [PubMed: 10581169]

28. Gallegos AM, Atshaves BP, Storey SM, et al. Gene structure, intracellular localization, and functional roles of sterol carrier protein-2. Prog Lipid Res 2001; 40: 498–563. [PubMed: 11591437]

29. McLean MP, Nanjo K, Irby RB, et al. Reduced hepatic sterol carrier protein-2 expression in the streptozotocin treated diabetic rat. Endocrine 1995; 3: 563–571. [PubMed: 21153133]

30. Zhang XL, Zhu QQ, Zhu L, et al. Safety and efficacy of anti-PCSK9 antibodies: a meta-analysis of 25 randomized, controlled trials. BMC Med 2015; 13: 123. [PubMed: 26099511]

31. Kastelein JJ, Ginsberg HN, Langslet G, et al. ODYSSEY FH I and FH II: 78-week results with alirocumab treatment in 735 patients with heterozygous familial hypercholesterolaemia. Eur Heart J 2015; 36: 2996–3003. [PubMed: 26330422]
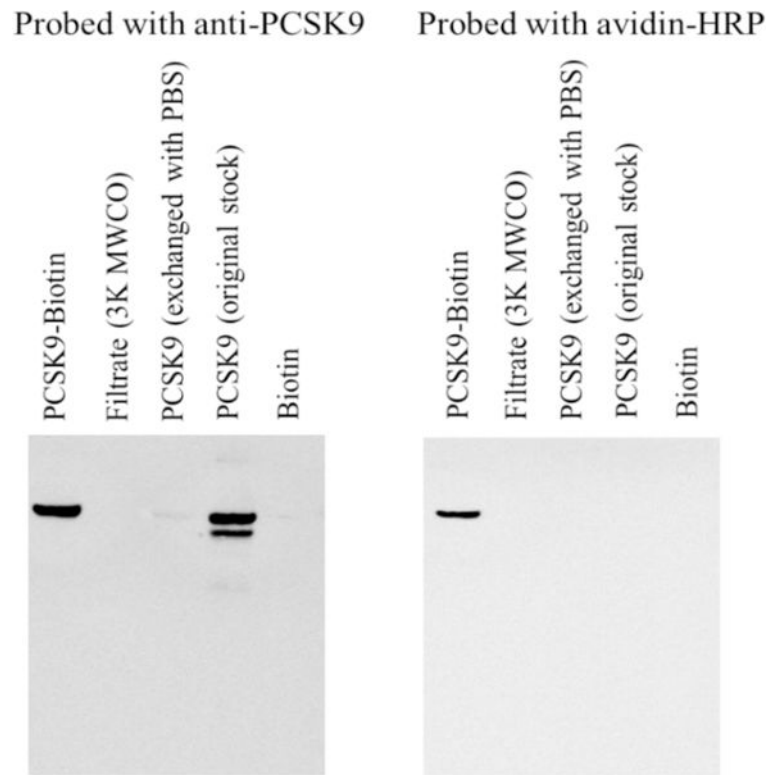
Figure 1:

Western blotting analysis of biotinylated PCSK9 before ProtoArray microarray analysis. Recombinant PCSK9 was biotinylated using the EZ Link™ Sulfo-NHS-SS Biotinylation kit as described in Materials and Methods. Electrophoresis followed by Western blotting analysis using either anti-PCSK9 specific antibody or avidin-HRP was done. Typical Western blots are shown. Biotinylated samples were purified using Zeba™ spin columns.
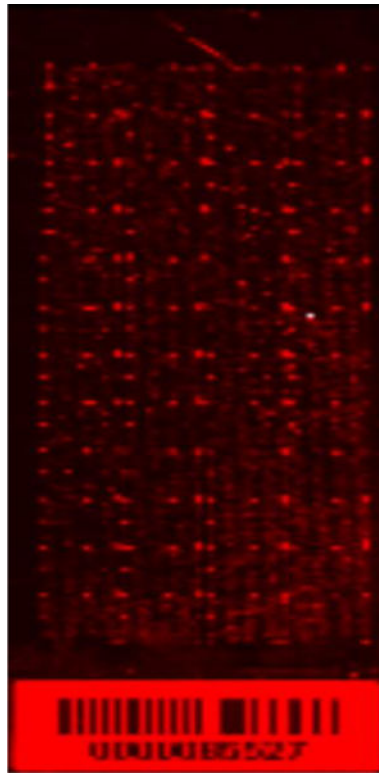
Figure 2:
ProtoArray Human Protein Microarray v5.0 probed with biotinylated rPCSK9 protein. Interactions were detected with streptavidin-Dylight 650 dye. Typical whole slide image is shown as acquired using a LiCor Odyssey scanner. This experiment was repeated three times.

Table 1:

Proteins identified by protein-protein interaction with biotinylated PCSK9 in a ProteinArray Human Protein Array.

Database ID	Mean Signal Used	Mean Z-Factor	Mean Z-Score	Mean CI P-Value	Mean CV	Description
NM_152389.1	6461	−1.94	7.06	0.023	0.64	coiled-coil domain containing 108 (CCDC108), transcript variant 2
NM_201564.1	5212	−0.26	5.28	0.033	0.33	synaptonemal complex central element protein 1 (SYCE1), transcript variant 2
BC059360.1	4557	−1.02	4.41	0.035	0.44	phosphoglucomutase 2-like 1 (PGM2L1)
BC005911.1	4896	−1.89	5.47	0.026	0.89	sterol carrier protein 2 (SCP2)
NM_145177.1	5777	−0.48	6.00	0.043	0.24	dehydrogenase/reductase (SDR family) X-linked (DHRSX)
BC005930.1	5206	−3.04	4.36	0.029	0.79	CD58 molecule (CD58)
BC019102.1	5923	−0.42	6.26	0.024	0.29	dedicator of cytokinesis protein 8
NM_016074.1	4165	−3.50	3.41	0.035	0.94	bolA homolog 1 (E. coli) (BOLA1)
BC021093.1	3762	−0.29	3.89	0.039	0.21	hippocampus abundant transcript-like protein 1
NM_022497.2	6189	0.10	6.49	0.026	0.22	mitochondrial ribosomal protein S25 (MRPS25), nuclear gene encoding mitochondrial protein
NM_020466.3	5388	−0.30	5.69	0.032	0.32	LYR motif-containing protein 2
NM_015959.1	4369	−0.12	4.53	0.032	0.14	thioredoxin domain-containing protein 14
NM_004527.2	4539	−0.72	4.73	0.028	0.36	mesenchyme homeobox 1 (MEOX1), transcript variant 1
BC050328.1	3464	−2.93	3.15	0.030	0.54	neuroblastoma breakpoint family, member 22 (pseudogene) (NBPF22P)
NM_016073.2	3932	−0.20	4.05	0.038	0.23	hepatoma-derived growth factor, related protein 3 (HDGFRP3)

This experiment was performed as described in Material and Methods.

Data from three independent arrays were analyzed using the ProtoArray Prospector Imager/Analyzer software 5.2.3 and the GAL file corresponding to each specific array.