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Nitrated Proteome in Human Embryonic Stem Cells

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Abstract : Post-translational modifications (PTMs) of proteins regulate self-renewal and differentiation in embryonic stem cells (ESCs). Nitration of tyrosine residues of proteins in ESCs modulates their downstream pathways, which can affect self-renewal and differentiation. However, protein tyrosine nitration (PTN) in ESCs has been rarely studied. We reviewed 23 nitrated sites in stem cell proteins. Functional enrichment analysis showed that these nitrated proteins are involved in signal transduction, cell adhesion and migration, and cell proliferation in ESCs. Comparison between the nitrated and known phosphorylated sites revealed that 7 nitrated sites had overlapping phosphorylated sites, indicating functional links of PTNs to their associated signaling pathways in ESCs. Therefore, nitrated proteome provides a basis for understanding potential roles of PTN in self-renewal and differentiation of ESCs.

Keywords : Tyrosine nitration, Nitrotyrosine enrichment, LC-MS/MS, Proteomics, Embryonic stem cells

Introduction

Nitration of tyrosine residues modulates the activity of proteins, thereby affecting their downstream processes.[1] Protein tyrosine nitration (PTN) plays key roles in regulating signaling and metabolic pathways under diseased conditions.[2-3] This PTN occurs through the action of reactive nitrogen species (RNS) under conditions of oxidative stress.[2] A growing body of evidence suggests that PTN can play roles in self-renewal and differentiation of embryonic stem cells (ESCs)[4-9] during early embryogenesis, nitric oxide (NO) synthases are expressed in the growing embryo, implying the presence of RNS gradients in the developing organs.[10-12] The exposure of ESCs to a high concentration of NO promotes differentiation of ESCs though repression of NANOG.[13-14] Nitration of pyruvate kinase isoform M2 mediated by RNS impairs proliferation of neural progenitor cells. These data collectively indicate that PTN can affect cellular processes related to self-renewal and differentiation of stem cells.[15-16] However, PTN in ESCs and its roles in self-renewal and differentiation of ESCs have been rarely studied.

Recently, mass spectrometry (MS)-based profiling methods have been employed to identify post-translationally modified proteins and understand their roles in stem cells.[17] For example, Brill *et al.* profiled phosphorylated proteomes of pluripotent cells and their differentiated cells and found that the phosphorylation of receptor tyrosine kinases plays significant roles in maintaining self-renewal of ESCs.[18] Therefore, MS-based profiling of nitrated proteomes in ESCs and their differentiated cells is critical to identify nitrated proteins and to understand their potential roles in self-renewal and differentiation of ESCs. However, liquid chromatography (LC)-MS/MS analysis of nitrated proteomes has been hampered due to a limited number of proteins undergoing PTN and low abundance of nitrated proteins.[10] To resolve this problem, we previously developed a fluorine-fluorine interaction-based affinity purification method to selectively isolate nitrated peptides.[19]

Here, we briefly reviewed a total of 23 nitrated sites from nitrated proteomes of stem cells and their differentiated cells. PTN exerts its functions by modulating phosphorylation of the nitrated tyrosine or neighboring serine/threonine residues.[20] Thus, the comparison between the detected nitrated and known phosphorylation sites can reveal functional implication of the nitrated sites.

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Furthermore, PTN modulates the activity of the proteins, thereby affecting activities of their interacting proteins in the downstream pathways. In this review, we reviewed these aspects to understand potential roles of the nitrated proteome in self-renewal and differentiation of stem cells. Therefore, our nitrated proteome provides a basis for understanding potential roles of PTN in self-renewal and differentiation of stem cells.

Backgrounds

Post-translational modifications (PTMs) modulate the activity of most of proteins and a variety of biological processes. Specifically protein tyrosine nitration (PTN) has been proposed as possible modification which regulates enzymatic activity of the modified proteins, not just as a marker for oxidative stress.[1] During PTN, nitrogen dioxide radical (NO_2•) is attached at the *ortho* position of hydroxyl group on tyrosine residues. Peroxynitrite ($ONOO^-$), which is produced by the combination of nitric oxide (NO•) and superoxide anion (O_2^-•) is necessarily needed to initiate this modification.[2-3] The reactive oxygen species (ROS) and the reactive nitrogen species (RNS) are key players to make proteins nitrated. The physicochemical properties of nitrated proteins have been reported to be affected as a consequence.[5] PTN had been considered as an irreversible process, but many evidences that nitration is reversible process have been suggested recently.[5-6] Understanding that nitration is reversible process controlled by certain conditions or factors, PTN may play as a regulator of several signal transductions or protein-protein interactions. Recently, Knyushko *et al.* reported that protein tyrosine nitration (PTN) of Ca-ATPase resulted in loss of Ca-ATPase activity and reduced transport function.[7] It was suggested that nitration provides a mechanism for down-regulation of ATP utilization by the Ca-ATPase.[8] It was reported that PTN was also associated with various pathological events, such as cardiovascular diseases and hypertension condition in kidney.[4] Under hypertensive condition, induced nitric oxide signaling may increase nitrosative and oxidative stress, which could generate high concentration of proteins containing 3-nitrotyrosine. Tyther *et al.* identified numerous nitrated proteins in the medulla of hypertensive rats.[11]

Method developments for analysis of nitrated peptides

Although the importance of PTN has been realized, analysis of PTN is difficult due to its low abundance and rapid turnover.[10] Hence effective enrichment method has been requested to analyze nitro-proteome. Some research groups have reported to identify nitrated proteins by the combined use of antibody against nitrotyrosine, 2D-PAGE and mass spectrometry.[13,15,17] Several publications on affinity enrichment methods for the low abundant nitrated peptides have been reported lately.[21-23] Most of these methods are based on immunoprecipitation and chemical conversions followed by affinity chromatography. We previously introduced an enrichment method of nitrated peptides based on metal chelating of bis-pyridines introduced to nitro-tyrosine residues. The resulting nitrated peptides with bis-pyridines formed a complex with Ni_2^+-NTA magnetic beads, so that the nitrated peptides or proteins could be isolated from the solution.[21] Recently, we developed another enrichment method for nitrated proteins by using modified fluorous solid phase extraction (FSPE).[19] FSPE utilized distinctive property of fluorine to isolate effectively fluorous-labeled peptides from non-labeled peptides mixture.[24-25]

Identification of nitrated proteomes from ESCs and their differentiated cells

Reactive nitrogen species (RNS) plays roles in escaping pluripotent cells from the self-renewing state and then initiating the differentiation. Furthermore, protein tyrosine nitration (PTN) generated from the RNS appears to promote the differentiation of pluripotent cells.[16] To investigate potential roles of PTN in ESCs, the nitrated proteome profiling is critical to identify nitrated proteins in ESCs and their differentiated cells, providing the difference in nitrated proteomes between undifferentiated ESCs and differentiated cells. Recent developments in high-throughput measurement technologies and equipment have offered new opportunities for research of human embryonic stem (ES) cells. The unique properties of stem cell were studied through a large amount of information from reported ES data. Many research groups focused on post-translational modifications (PTM) of proteins in ES cells. Especially, Jeroen *et al*. reported that effect of phosphorylation on differentiation of hES cells.[26]

PTN has been reported to promote differentiation of pluripotent cells.[16] To investigate this characteristic of PTN at the proteome level, we first selected a total of 23 nitrated peptides in ES cells and their differentiated cells (Table 1) from previous results and our own results.

Cellular processes associated with nitrated proteins

PTN often occurs in signaling molecules, thus modulating activities of their associated signaling pathways.[27] To examine what cellular processes are associated with PTN in ESCs and their differentiated cells, we performed function enrichment analysis to identify GO biological processes (BPs), molecular functions (MFs), and cellular components (CCs) enriched by the 23 nitrated proteins (NPs) using DAVID software.[28] The GOBP most significantly represented by the 23 NPs were 'cell

Table 1. The 23 nitrated peptides detected from stem cells. Modifications in the sequences are denoted by @, the perfluorination on tyrosine resideue; *, oxidation on methionine;] and #, acetylation at the N-terminal and lysine residues, respectively. "ST" means SpectraST search results for all MS/MS spectra[33] against constructed database of nitroproteins from huh 7 cell lines.[19]

Uniprot ID	Symbol	Description	Peptide Sequence	XCorr
O95171	SCEL	sciellin	Q]IHPPK#PGVY@TETNR	2.053
O95071	UBR5	ubiquitin protein ligase E3 component n-recognin 5	P]SQGLY@SSSASSGK#	2.074
Q86VZ4	LRP11	low density lipoprotein receptor-related protein 11	P]ITSEESDYLINGMY@L	2.083
Q9H7Z7	PTGES2	Prostaglandin E synthase 2	E]DLY@EAADK	2.094
Q9HBL0	TNS1	tensin 1	S]K#HGGNY@LLFNLSER	2.127
Q92523	CPT1B	Carnitine O-palmitoyltransferase 1, muscle isoform	L]GNIIHAM*IM*Y@RR	2.278
Q9BWW9	APOL5	apolipoprotein L, 5	A]IQGIKDLHAY@QMAK#	2.328
Q9Y680	FKBP7	FK506 binding protein 7	Q]LSKAEINLY@LQR	2.501
O00160	MYO1F	myosin IF	Y]IMGY@ISK#VSGGGEK#	2.568
Q5BJE1	C18orf34	chromosome 18 open reading frame 34	H]LKNIYK#EAY@R	2.22
Q9HCS4	TCF7L1	transcription factor 7-like 1 (T-cell specific, HMG-box)	T]Y@LQM*KWP LLDVPSSATVK	2.264
Q2TB22	RASAL2	RAS protein activator like 2	S]RFQTITILPM*EQY@K	2.276
P42263	GRIA3	glutamate receptor, ionotrophic, AMPA 3	S]K#IAVY@EK MWSYM*K#	2.333
P55196	MLLT4	afadin	D]Y@EPPSPSPA PGAPPPPPQR	2.454
P32004	L1CAM	L1 cell adhesion molecule	I]NGIPVEELAKDQKY@R	2.527
O94832	MYO1D	myosin ID	H]Y@AGDVVYSVIGF IDKNKDTLFQDFK#	2.528
Q8TE04	PANK1	pantothenate kinase 1	P]YCLDNPY@PMLLVN MGSGVSILAVYSK#	2.59
A6NMZ7	COL6A6	collagen, type VI, alpha 6	D]LSM*FSQNM*TH IIKDVIK#Y@K	2.787
P17024	ZNF20	zinc finger protein 20	A]FSY@LDSFQSH DK#ACTKEK	2.803
Q13606	OR5I1	olfactory receptor, family 5, subfamily I, member 1	I]ISVFY@TIFIPV LNPLIY@SLR	3.388
O14974	PPP1R12A	protein phosphatase 1, regulatory (inhibitor) subunit 12A	L]AY@VAPTIPR	ST
Q969Z0	TBRG4	transforming growth factor beta regulator 4	P]FSLTKDVLLD VAYAY@GK#	ST
Q9UHC6	CNTNAP2	contactin associated protein-like 2	C]TCDETGYSGATC*H NSIYEPSC*EAY@K	ST

adhesion' and 'cell surface receptor linked signal transduction', consistent to the known association of PTN with signaling transduction (Figure 1A). The GOBPs represented by the NPs include the ones related to 1) cell adhesion, 2) cell surface receptor linked signal transduction 3) regulation of transcription, and 4) molecular transports (lipid transport and ion transport). The GOMFs enriched by the NPs included ATP, ion, actin, and nucleotide binding, as well as DNA binding, which are closely related to the enriched GOBPs (Figure 1B). Furthermore, the NPs are localized significantly to membrane, non-membrane-bounded organelle, and cytoskeleton, where signaling molecules, transport-related molecules, and actin binding molecules are localized, respectively (Figure 1C). These results collectively indicate that the NPs in ESCs and their differentiated cells can play roles in actin cytoskeleton

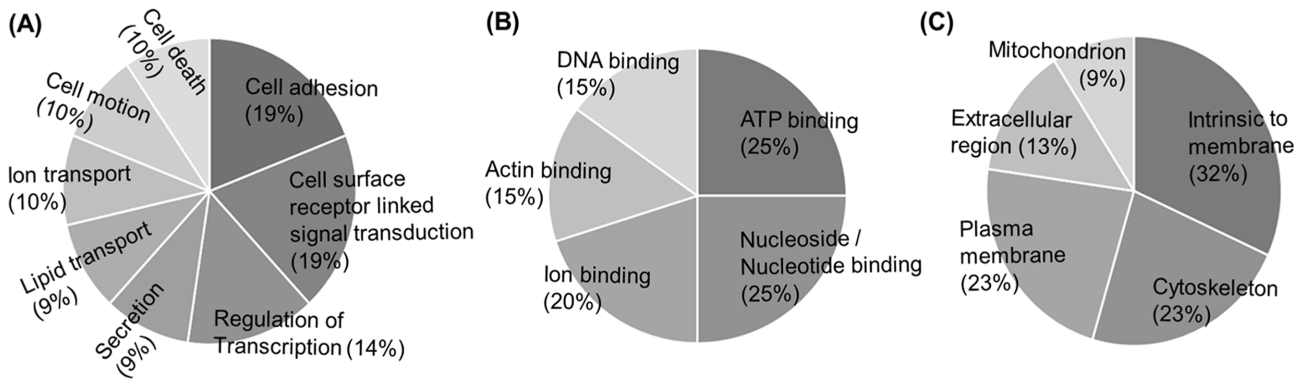

Figure 1. GO enrichment analysis of the 23 nitrated proteins. GO biological processes (GOBPs; A), molecular functions (GOMFs, B), and cellular components (GOCCs; C) enriched by the 23 nitrated proteins with P-values less than 0.05 from DAVID software.

Table 2. The phosphorylation sites from the two resources are presented in the columns labeled in Phosphosite Plus,[29] Phosida,[30] and Phospho.ELM.[31]

Uniprot	Symbol	nY	Phosphosite Plus	Phosida	PhosphoELM
O95171	SCEL	Y219	S195, S196, S199, S200, Y219, T220, T222, S263	NA	NA
O95071	UBR5	Y2075	S2026, S2028, T2030, S2071, Y2075, S2076, S2103	S2026, S2028, T2030	S2028
Q86VZ4	LRP11	Y499	S491, Y493, Y499	NA	NA
Q9H7Z7	PTGES2	Y308	S260, Y263, Y280, Y287, S290, Y308	S260, Y263	NA
Q9HBL0	TNS1	Y55	T68, T84, S92	NA	NA
Q92523	CPT1B	Y284	S330	NA	NA
Q9BWW9	APOL5	Y246	Y246	NA	NA
Q9Y680	FKBP7	Y172	NA	NA	NA
O00160	MYO1F	Y126	Y122	NA	NA
Q5BJE1	C18orf34	Y503	NA	NA	NA
Q9HCS4	TCF7L1	Y148	NA	NA	NA
Q9UJF2	RASAL2	Y431	Y381, Y389	NA	NA
P42263	GRIA3	Y699	NA	NA	NA
P55196	MLLT4	Y1692	Y1656, Y1657, S1658, Y1692, S1696, S1698, S1721	NA	S1681
P32004	L1CAM	Y382	Y418, Y420	NA	NA
O94832	MYO1D	Y508	S493, Y514, Y536	NA	NA
Q8TE04	PANK1	Y408	T451, T455	NA	NA
A6NMZ7	COL6A6	Y209	Y247	NA	NA
P17024	ZNF20	Y154	T128, S132, S133, Y143, S194	NA	NA
Q13606	OR5I1	Y280	Y261	NA	NA
O14974	PPP1R12A	Y496	Y446, T453, T464, S470, S472, S473, S477, S478, S479, Y496, T500, S507, T508, S509, S520, S525, T529, S541, S542, S547,	NA	T443, S445, Y446, S473, S477, S478, S507, T508, S509
Q969Z0	TBRG4	Y302	Y300	NA	NA
Q9UHC6	CNTNAP2	Y602	T638, S641	NA	NA

reorganization, molecular transport, and transcription, in addition to their known roles in signal transduction.

Relationships between nitrated and phosphorylated sites

Nitration competitively inhibits phosphorylation at the same tyrosine residue.[5] Further, nitration at a tyrosine residue can modulate phosphorylation of neighboring sites.[20] To examine the potential effect of nitration on signaling pathways, we first collected phosphorylation sites for the 23 NPs from the following three PTM databases (Phosphosite Plus,[29] Phosida,[30] Phospho.ELM[31]). Among the 23 NPs, 19 proteins were phosphorylated (Table 2). Seven of the 23 nitrated proteins (SCEL, UBR5, LRP11, PTGES2, APOL5, MLLT4 AND PPP1R12A) shared the nitrated and phosphorylated tyrosine residues (Table 2). PPP1R12A, a myosin phosphotase regulates cell-cell interactions and cell migration, as a downstream regulator of Rho signaling pathway, during early differentiation of hESCs,[32] thereby promoting self-renewal of stem cells. These data imply that the myosin and actin modulating proteins (PPP1R12A) may regulate self-renewal and differentiation of hESCs.

To investigate the effect of nitration on neighboring phosphorylation, we also examined the distribution of the distances between the nitration and phosphorylation sites (Table 2). How close nitration and phosphorylation should take place to modulate each other. Among the 23 proteins, 14 proteins (61%) had the distances between nitration and phosphorylation sites less than 30 AAs (Table 2). This result suggests that phosphorylation can modulate nitration vice versa when they take place apart up to 30 amino acids (AAs). In particular, four proteins (MYO1F, MYO1D, ZNF20 and TBRG4 had phosphorylation sites nearby the nitrated tyrosine residues within 10 amino acids (Table 2). In addition to PPP1R12A and CFL1 aforementioned, MYO1F and MYO1D, other myosin complex proteins also contribute to cytoskeleton reorganization by binding to actin cytoskeleton. The GO enrichment analysis (Figure 1A) showed a potential role of the nitrated proteins in cell adhesion. Taken together, these data indicate that phosphorylation of the three myosin and actin binding proteins (PPP1R12A, MYO1D, and MYO1F) can be collectively modulated by PTN and phosphorylation, thereby affecting the activity of the actin cytoskeleton reorganization network.

Conclusions

In this review, we analyzed nitrated proteome profiles that can provide lists of NPs in ESCs and their differentiated cells and the changes in the nitration along the differentiation. Furthermore, the integrated analysis of the nitrated proteome with known phosphorylation data showed the possibility that the two PTMs can be cross-modulated by each other, which can affect self-renewal and differentiation of ESCs. Similarly, the nitrated proteome can be further integrated with various kinds of data (e.g. other PTMs) to understand diverse aspects of functional roles of nitration in self-renewal and differentiation. Therefore, nitrated proteome provides a basis for understanding potential roles of PTN in self-renewal and differentiation of ESCs.

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Reference

1. Souza, J. M.; Peluffo, G.; Radi, R. *Free Radical Bio. Med.* **2008**, 45, 357.
2. Radi, R. *P. Natl. A. Sci. USA* **2004**, 101, 4003.
3. Lee, J. R.; Kim, J. K.; Lee, S. J.; Kim, K. P. *Arch. Pharm. Res.* **2009**, 32, 1109.
4. Turko, I. V.; Murad, F. *Pharmacol. Rev.* **2002**, 54, 619.
5. Abello, N.; Kerstjens, H. A.; Postma, D. S.; Bischoff, R. *J. Proteome Res.* **2009**, 8, 3222.
6. Csibi, A.; Communi, D.; Muller, N.; Bottari, S. P. *PloS one* **2010**, 5, e10070.
7. Knyushko, T. V.; Sharov, V. S.; Williams, T. D.; Schoneich, C.; Bigelow, D. J. *Biochemistry* **2005**, 44, 13071.
8. Xu, S.; Ying, J.; Jiang, B.; Guo, W.; Adachi, T.; Sharov, V.; Lazar, H.; Menzoian, J.; Knyushko, T. V.; Bigelow, D.; Schoneich, C.; Cohen, R. A. *Am. J. Physiol-Heart C.* **2006**, 290, H2220.
9. Venkatesan, A.; Uzasci, L.; Chen, Z.; Rajbhandari, L.; Anderson, C.; Lee, M. H.; Bianchet, M. A.; Cotter, R.; Song, H.; Nath, A. *Mol. Brain* **2011**, 4, 28.
10. Ischiropoulos, H. *Biochem. Bioph. Res. Co.* **2003**, 305, 776.
11. Tyther, R.; Ahmeda, A.; Johns, E.; Sheehan, D. *Proteomics* **2007**, 7, 4555.
12. Bloch, W.; Fleischmann, B. K.; Lorke, D. E.; Andressen, C.; Hops, B.; Hescheler, J.; Addicks, K. *Cardiovasc. Res.* **1999**, 4, 675.
13. Yeo, W. S.; Lee, S. J.; Lee, J. R.; Kim, K. P. *BMB Rep.* **2008**, 41, 194.
14. Tejedo, J. R.; Tapia-Limonchi, R.; Mora-Castilla, S.; Cahuana, G. M.; Hmadcha, A.; Martin, F.; Bedoya, F. J.; Soria, B. *Cel. Death Dis.* **2010**, 1, e80.
15. Zhan, X.; Desiderio, D. M. *Method. Mol. Cell. Biol.* **2009**, 566, 137.
16. Kim, S. J.; Lim, M. S.; Kang, S. K.; Lee, Y. S.; Kang, K. S. *Cell. Res.* **2008**, 18, 686.

17. Franze, T.; Weller, M. G.; Niessner, R.; Poschl, U. *The Analyst* **2004**, 12, 589.
18. Brill, L. M.; Xiong, W.; Lee, K. B.; Ficarro, S. B.; Crain, A.; Xu, Y.; Terskikh, A.; Snyder, E. Y.; Ding, S. *Cell Stem Cell* **2009**, 5, 204.
19. Kim, J. K.; Lee, J. R.; Kang, J. W.; Lee, S. J.; Shin, G. C.; Yeo, W. S.; Kim, K. H.; Park, H. S.; Kim, K. P. *Anal. Chem.* **2011**, 83, 157.
20. Rayala, S. K.; Martin, E.; Sharina, I. G.; Molli, P. R.; Wang, X.; Jacobson, R.; Murad, F.; Kumar, R. *P. Natl. Acad. Sci. USA* **2007**, 104, 19470.
21. Lee, J. R.; Lee, S. J.; Kim, T. W.; Kim, J. K.; Park, H. S.; Kim, D. E.; Kim, K. P.; Yeo, W. S. *Anal. Chem.* **2009**, 81, 6620.
22. Zhang, Q.; Qian, W. J.; Knyushko, T. V.; Clauss, T. R.; Purvine, S. O.; Moore, R. J.; Sacksteder, C. A.; Chin, M. H.; Smith, D. J.; Camp, D. G., 2nd; Bigelow, D. J.; Smith, R. D. *J. Proteome Res.* **2007**, 6, 2257.
23. Nikov, G.; Bhat, V.; Wishnok, J. S.; Tannenbaum, S. R. *Anal. Biochem.* **2003**, 320, 214.
24. Brittain, S. M.; Ficarro, S. B.; Brock, A.; Peters, E. C. *Nat. Biotechnol.* **2005**, 2, 463.
25. Go, E. P.; Uritboonthai, W.; Apon, J. V.; Trauger, S. A.; Nordstrom, A.; O'Maille, G.; Brittain, S. M.; Peters, E. C.; Siuzdak, G. *J. Proteome Res.* **2007**, 6, 1492.
26. Van Hoof, D.; Munoz, J.; Braam, S. R.; Pinkse, M. W.; Linding, R.; Heck, A. J.; Mummery, C. L.; Krijgsveld, J., Phosphorylation dynamics during early differentiation of human embryonic stem cells. *Cell stem cell* **2009**, 5, 214.
27. Monteiro, H. P. *Free Radical Bio. Med.* **2002**, 33, 765.
28. Huang da, W.; Sherman, B. T.; Lempicki, R. A. *Nat. Protoc.* **2009**, 4, 44.
29. Hornbeck, P. V.; Chabra, I.; Kornhauser, J. M.; Skrzypek, E.; Zhang, B. *Proteomics* **2004**, 4, 1551.
30. Gnad, F.; Gunawardena, J.; Mann, M. *Nucleic Acids Res.* **2011**, 39, D253.
31. Dinkel, H.; Chica, C.; Via, A.; Gould, C. M.; Jensen, L. J.; Gibson, T. J.; Diella, F. *Nucleic Acids Res.* **2011**, 39, D261.
32. Harb, N.; Archer, T. K.; Sato, N. *PloS one* **2008**, 3, e3001.
33. Bolstad, B. M.; Irizarry, R. A.; Astrand, M.; Speed, T. P. *Bioinformatics* **2003**, 19, 185.