# Housekeeping Proteins: Limitations as References During Neuronal Differentiation

Zafira Castaño* and Robert M. Kypta

*CIC bioGUNE (Center for Cooperative Research in Biosciences), Spain*

**Abstract:** The use of "Housekeeping proteins" as loading controls in western blots requires that they do not change their level of expression. However, deviations from this rule have been observed. This study investigated the expression of the commonly used proteins HSP60, βI-tubulin, β-actin and GAPDH by western blot during retinoic acid-induced differentiation of the embryonal carcinoma NTera-2 and the neuroblastoma SH-SY5Y cell lines. β-actin was found to be a poor loading control, increasing significantly during differentiation, whereas HSP60 and GAPDH were the more evenly expressed of those analysed. Our results underline the importance of selecting accurate loading controls in this biological context.

**Keywords**: Housekeeping protein, western blotting, NTera-2/clone D1, SH-SY5Y, retinoic acid, β-actin.

## INTRODUCTION

Two of the most commonly used human cell lines for studies of neuronal differentiation are NTera-2/clone D1 (NT2) and SH-SY5Y. NT2 is a human embryonal carcinoma (EC) cell line that differentiates after treatment with retinoic acid (RA) [1-6]. More recently, NT2 cells have been used as a model system to study dopaminergic differentiation of human embryonic stem (hES) cells [7]. SH-SY5Y is a neuroblastic subclone of the neuroblastoma cell line SK-N-SH [8]. SH-SY5Y cells withdraw from the cell cycle and express a distinct neuronal phenotype when treated with RA [9], and are used extensively as a cell culture model for neuronal differentiation, survival and apoptosis [9-13].

A systematic study of consistently expressed housekeeping proteins during the differentiation of NT2 or SH-SY5Y cells has not been described. Proteins that have been used as internal controls include the mitochondrial protein HSP60, the cytoskeletal proteins βI-tubulin and β-actin, and the metabolic enzyme GAPDH [14-16]. There is a growing body of evidence that some commonly used housekeeping proteins are inadequate internal standards because they are themselves affected by drug treatments, cell cycle phase, differentiation or cell proliferation [17-21]. Neuronal differentiation is a dynamic process involving dramatic changes in cell protein expression. Therefore, we sought to identify consistently expressed cellular proteins to use as controls in protein expression studies during RA-induced differentiation of NT2 and SH-SY5Y cells.

## MATERIAL AND METHODS

### Cell Culture

SH-SY5Y cells (ATCC, CRL-2266) were cultured in 1:1 F-12K/MEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 2 mM l-glutamine and 1% non-essential amino acids (NEAA) (all reagents were from Invitrogen). To induce differentiation, 5 x $10^5$ cells were seeded in 10 cm tissue culture dishes and cultured for up to 4 days with 10 μM all-trans retinoic acid (RA) (Sigma-Aldrich). NT2 cells (from P. Andrews, Edinburgh) were cultured in DMEM/F12 (high glucose formulation) supplemented with 10% FCS and 1% penicillin/streptomycin. NT2 cells were subcultured using a cell scraper (Sarsted, Newton, NC). To induce differentiation, 4 x $10^5$ cells were seeded in 10 cm tissue culture dishes and grown for up to 28 days with 10 μM RA. As controls, both cell lines were treated with vehicle (0.1% DMSO).

### *Western Blot* Analysis

Lysates were prepared using modified RIPA lysis buffer (0.5% sodium deoxycholate, 1% Triton X-100, 20 mM Tris pH 8, 0.1% SDS, 100 mM NaCl, 50 mM NaF, 1 mM EDTA and protease inhibitor cocktail (Roche)). Protein concentrations were determined using a modified Bradford assay (Bio-Rad). Proteins (14 μg/lane) were separated on 10% polyacrylamide gels and transferred to Immobilon-P membrane (Millipore), using a Trans-Blot semi-dry transfer apparatus (Bio-Rad). Blots were blocked in blocking buffer (3% BSA in TBS-T) for 30 min at room temperature. Blots were probed with primary antibody diluted in blocking buffer for 1 h. The primary antibodies used were rabbit anti-HSP60 (H-300, Santa Cruz Biotechnology), mouse anti-βI-tubulin (clone 2-28-33, Sigma), rabbit anti-β-actin (Abcam) and mouse anti-GAPDH (clone 71.1, Sigma), all diluted 1:5000, and mouse anti-βIII-tubulin (TU-20, Chemicon) diluted 1:3000. After washing with TBS-T, blots were incubated for 30 min with HRP-conjugated secondary antibodies (Jackson Inmunoresearch Laboratories) diluted 1:10000 in blocking buffer and washed again in TBS-T. Detection of proteins was by chemiluminescence (ECL, Amersham Biosciences, UK), followed by analysis in a Chemidoc XRS machine using Quantity One-4.6.2 1-D analysis software (Bio-Rad). Blots were exposed for different times, and exposures in the linear range of signal were selected for densitometric evaluation.

*Address correspondence to this author at the CIC bioGUNE, Cell Biology and Stem Cells Unit, Parque Tecnologico de Bizkaia, Edificio 801A, 48160 Derio, Bizkaia, Spain; Tel: (34) 94 406 1328; Fax: (34) 94 406 1301; E-mail: zcastano@cicbiogune.es

1874-0820/08 

### Statistical Analysis

Each experiment was repeated at least twice using samples from 2 independent experiments. Changes in protein expression were tested using one-way ANOVA followed by Dunnett´s test.

## RESULTS AND DISCUSSION

Western blotting revealed single bands at the expected molecular masses of approximately 50 kDa (βIII-tubulin), 60 kDa (HSP60), 50 kDa (βI-tubulin), 42 kDa (β-actin) and 36 kDa (GAPDH). Fig. (**1**) shows the expression levels of these proteins during differentiation of SH-SY5Y cells. Neuronal differentiation of SH-SY5Y cells becomes morphologically evident at 4 days RA treatment (Fig. **1a**), concomitant with expression of the neuronal marker, βIII-tubulin (Fig. **1b**, top pannel). The same blot was probed for housekeeping proteins (Fig. **1b**, lower panels). HSP60 and GAPDH were similarly expressed in undifferentiated (DMSO treated cells) and differentiated samples (4 days RA treated cells), whereas βI-tubulin, and, more significantly, β-actin levels increased dur-

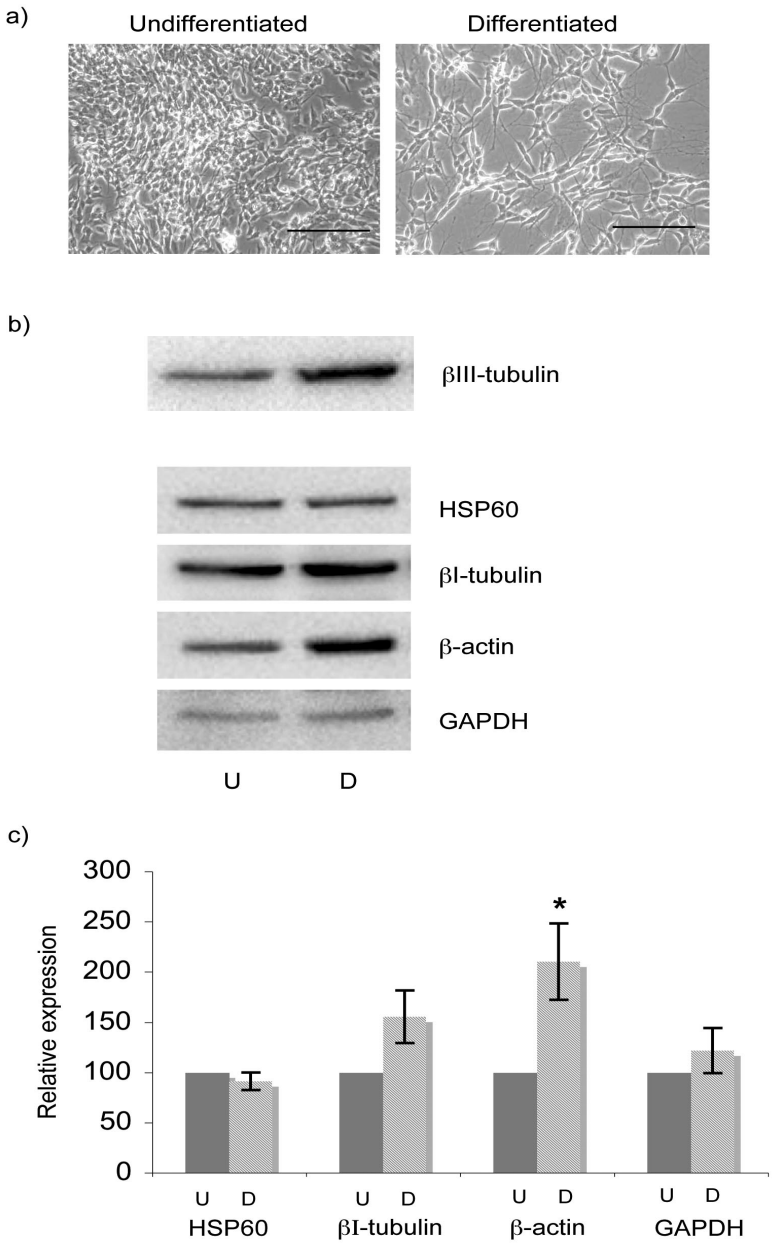


**Fig. (1). Housekeeping protein expression levels during SH-SY5Y cell differentiation.**

(**a**) SH-SY5Y cell morphology in undifferentiated cells (DMSO treated cells) and in differentiated cells after treatment with 10 μM RA for 4 days. (**b**) Analysis of extracts from undifferentiated (U) and differentiated (D) SH-SY5Y cells treated with vehicle (DMSO) or with $10^{-5}$ M RA for 4 days. Top panel: western blot showing βIII-tubulin, a neuron-specific protein; lower panels: western blots showing HSP60, βI-tubulin, β-actin and GAPDH. (**c**) Graphical representation of densitometry data from HSP60, βI-tubulin, β-actin and GAPDH in undifferentiated (U) and differentiated (D) samples. Bars represent means ± S.E.M. of OD values expressed as percentages of control for n=4. (*) $p<0.05$ for differentiated vs. control (one-way ANOVA and Dunnet´s test). Scale bar: 500 μm.

ing differentiation. The densitometric data are presented as a percentage of the undifferentiated signal in Fig. (**1c**). The expression levels of HSP60 and GAPDH were similar in undifferentiated and differentiated cells, with no significant variation (HSP60 levels in differentiated cells were 93 ± 8 % of control; GAPDH levels were 121 ± 22 % of control) (mean ± S.E.M.). In contrast, βI-tubulin levels showed a trend for an increase in differentiated cells (155 ± 26 % of control) and β-actin levels increased significantly (210 ± 38 % of control).

A similar analysis for NT2 cells is shown in Fig. (**2**). At 28 days RA treatment (differentiated cells) there was a clear change in NT2 morphology with the appearance of neuron-like cells compared with the DMSO treated cells (undifferen-

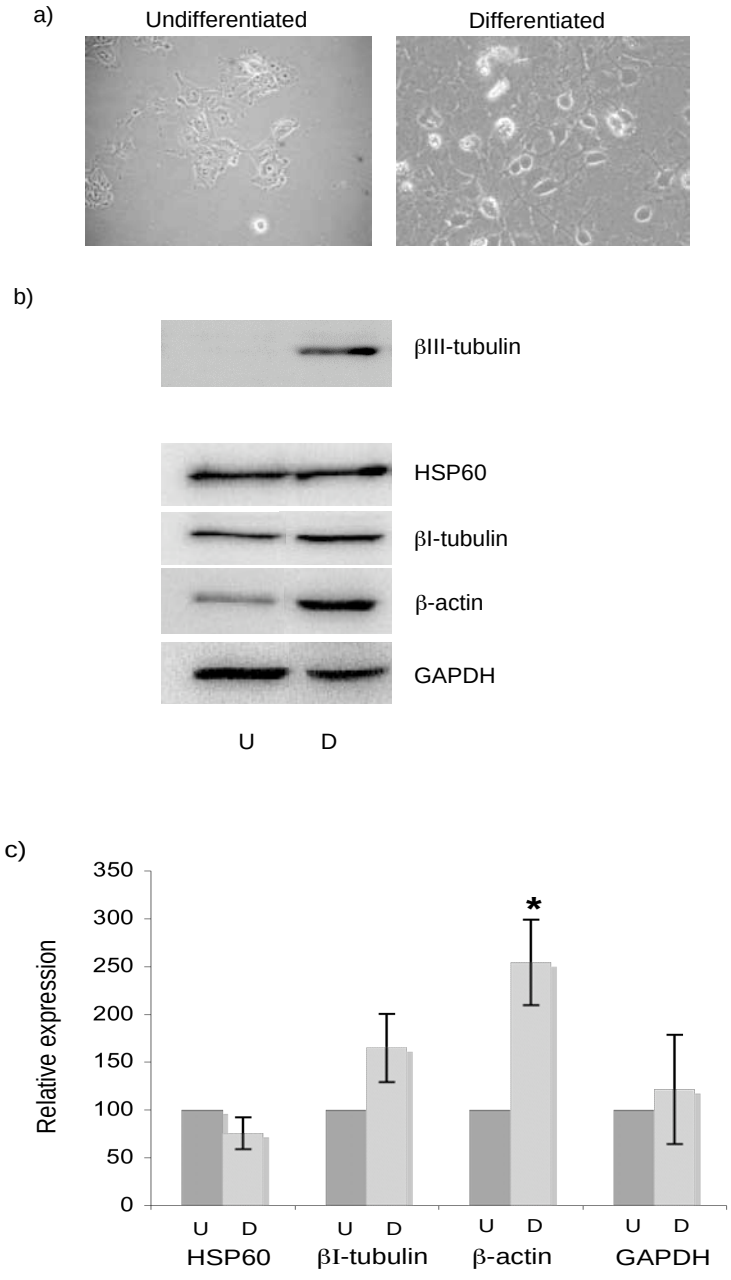


**Fig. (2). Housekeeping protein expression levels during NT2 cell differentiation.**

(**a**) NT2 cell morphology in undifferentiated cells (DMSO treated cells) and in differentiated cells after treatment with 10 μM RA for 28 days. (**b**) Analysis of extracts from undifferentiated (U) and differentiated (D) NT2 cells treated with DMSO or with $10^{-5}$ M RA for 28 days. Top panel: western blot showing βIII-tubulin, a neuron-specific protein; lower panels: western blots showing HSP60, βI-tubulin, β-actin and GAPDH. (**c**) Graphical representation of densitometry data from HSP60, βI-tubulin, β-actin and GAPDH in undifferentiated (U) and differentiated (D) samples. Bars represent means ± S.E.M. of OD values expressed as percentages of control for n=4. (*) $p<0.05$ for differentiated vs. control (one-way ANOVA and Dunnet´s test). Scale bar: 500 μm.

tiated cells) (Fig. **2a**). Differentiation correlated with the expression of βIII-tubulin (Fig. **2b**, top pannel). The expression levels of housekeeping proteins from this blot are shown in Fig. (**2b**) (lower panels). Once again, HSP60 and GAPDH were evenly expressed during differentiation, and βI-tubulin levels increased to a small extent, although this was not statistically significant. As in SH-SY5Y cells, β-actin levels were significantly increased highly upon RA treatment. The densitometry data are presented as a percentage of the undifferentiated values in Fig. (**1c**). Overall, differentiated cells had no significant increase in the levels of HSP60 (75 ± 17 % of control) and GAPDH (122 ± 57 %) in comparison to undifferentiated cells, whereas the level of βI-tubulin (165 ± 35 %) increased, although this increase did not reach statistical significance, and β-actin levels (254 ± 45 %) increased significantly in differentiated cells.

To summarise, the level of β-actin increases upon RA treatment in both SH-SY5Y and NT2 cells. This is probably because β-actin is found at high levels in axons and dendrites, which are likely to be more abundant in RA-treated cells. β-actin has been used as a loading control in several NT2 and SH-SY5Y cell differentiation studies [14, 22-26]. Our results suggest that using β-actin as a loading control will lead to an underestimation of the levels of other protein(s) under study in the differentiated cell population.

## CONCLUSION

We have shown that the expression levels of proteins currently used as internal controls in neuronal differentiation studies can vary significantly. As a consequence, the use of housekeeping proteins as internal standards should be examined carefully in relation to the cell or experimental conditions. Our data suggest that HSP60 or GAPDH should be used as a loading control for proteomic studies of NT2 and SH-SY5Y cell differentiation.

## ACKNOWLEDGEMENTS

We are grateful to Professor Peter Andrews (University of Sheffield, UK) for NTera-2 cells. This work was funded by the Department of Industry, Tourism and Trade of the Government of the Autonomous Community of the Basque Country (Etortek Research Programs 2005/ 2006) and the Innovation Technology Department of the Basque Country.

## ABBREVIATIONS

EC = embryonal carcinoma

NT2 = NTera-2/clone D1

RA = retinoic acid

## REFERENCES

[1] Almstrup K, Hoei-Hansen CE, Wirkner U, *et al*. Embryonic stem cell-like features of testicular carcinoma in situ revealed by genome-wide gene expression profiling. Cancer Res 2004; 64: 4736-43.

[2] Andrews PW. Human teratocarcinomas. Biochim Biophys Acta 1988; 948: 17-36.

[3] Andrews PW. Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line *in vitro*. Dev Biol 1984; 103: 285-93.

[4] Andrews PW, Matin MM, Bahrami AR, Damjanov I, Gokhale P, Draper JS. Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin. Biochem Soc Trans 2005; 33: 1526-30.

[5] Skotheim RI, Lind GE, Monni O, *et al*. Differentiation of human embryonal carcinomas *in vitro* and *in vivo* reveals expression profiles relevant to normal development. Cancer Res 2005; 65: 5588-98.

[6] Walsh J, Andrews PW. Expression of Wnt and Notch pathway genes in a pluripotent human embryonal carcinoma cell line and embryonic stem cell. APMIS 2003; 111: 197-210.

[7] Schwartz CM, Spivak CE, Baker SC, *et al.* NTera2: a model system to study dopaminergic differentiation of human embryonic stem cells. Stem Cells Dev 2005; 14: 517-34.

[8] Biedler JL, Spengler BA. A novel chromosome abnormality in human neuroblastoma and antifolate-resistant Chinese hamster cell lives in culture. J Natl Cancer Inst 1976; 57: 683-95.

[9] Ross RA, Spengler BA, Biedler JL. Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J. Natl Cancer Inst 1983; 71: 741-7.

[10] Encinas M, Iglesias M, Llecha N, Comella JX. Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. J Neurochem 1999; 73: 1409-21.

[11] Grynspan F, Griffin WB, Mohan PS, Shea TB, Nixon RA. Calpains and calpastatin in SH-SY5Y neuroblastoma cells during retinoic acid-induced differentiation and neurite outgrowth: comparison with the human brain calpain system. J Neurosci Res 1997; 48: 181-91.

[12] Pahlman S, Ruusala AI, Abrahamsson L, Mattsson ME, Esscher T. Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. Cell Differ 1984; 14: 135-44.

[13] Tosetti P, Taglietti V, Toselli M. Functional changes in potassium conductances of the human neuroblastoma cell line SH-SY5Y during *in vitro* differentiation. J Neurophysiol 1998; 79: 648-58.

[14] Mojsin M, Grujicic NK, Nikcevic G, Krstic A, Savic T, Stevanovic M. Mapping of the RXRalpha binding elements involved in retinoic acid induced transcriptional activation of the human SOX3 gene. Neurosci Res 2006; 56: 409-18.

[15] Moroni RF, Riasi SD, Colapietro D, Larizza L, Beghini A. Distinct expression pattern of microtubule-associated protein/microtubule affinity-regulating kinase 4 in differentiated neurons. Neuroscience 2006; 143: 83-94.

[16] Vignali G, Niclas J, Sprocati MT, Vale RD, Sirtori C, Navone F. Differential expression of ubiquitous and neuronal kinesin heavy chains during differentiation of human neuroblastoma and PC12 cells. Eur J Neurosci 1996; 8: 536-44.

[17] Murphy CL, Polak JM. Differentiating embryonic stem cells: GAPDH, but neither HPRT nor beta-tubulin is suitable as an internal standard for measuring RNA levels. Tissue Eng 2002; 8: 551-9.

[18] Murphy RM, Watt KK, Cameron-Smith D, Gibbons CJ, Snow RJ. Effects of creatine supplementation on housekeeping genes in human skeletal muscle using real-time RT-PCR. Physiol Genomics 2003; 12: 163-74.

[19] Oliveira JG, Prados RZ, Guedes AC, Ferreira PC, Kroon EG. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase is inappropriate as internal control in comparative studies between skin tissue and cultured skin fibroblasts using Northern blot analysis. Arch Dermatol Res 1999; 291: 659-61.

[20] Schmittgen TD, Zakrajsed BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 2000; 46: 69-81.

[21] Thellin O, Zorzi W, Lakaye B, *et al.* Housekeeping genes as internal standards: use and limits. J Biotechnol 1999; 75: 291-5.

[22] De los Santos M, Zambrano A, Sánchez-Pacheco A, Aranda A. Histone deacetylase inhibitors regulate RAR{beta} expression in neuroblastoma cells by both transcriptional and post-transcriptional mechanis. Mol Endocrinol 2007; 21: 2416-26.

[23] Freemantle SJ, Vaseva AV, Ewings KE, *et al.* Repression of cyclin D1 as a target for germ cell tumors. Int J Oncol 2007; 30: 333-40.

[24] McMillan CR, Sharma R, Ottenhof T, Niles LP. Modulation of tyrosine hydroxylase expression by melatonin in human SH-SY5Y neuroblastoma cells. Neurosci Lett 2007; 419: 202-6.

[25] Megiorni F, Indovina P, Mora B, Mazzilli MC. Minor expression of fascin-1 gene (FSCN1) in NTera2 cells depleted of CREB-binding protein. Neurosci Lett 2005; 381: 169-74.

[26] Pan J, Kao Y-L, Joshi S, Jeetendran S, DiPette D, Singh US. Activation of Rac1 by phosphatidylinositol 3-kinase *in vivo*: Role in activation of mitogen-activated protein kinase (MAPK) pathways and retinoic acid-induced neuronal differentiation of SH-SY5Y cells. J Neurochem 2005; 93: 571-83.

Received: June 09, 2008 Revised: July 25, 2008 Accepted: August 18, 2008