

## MicroRNA-9 represses sirtuin 1 (SIRT1) in human keratinocytes

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**Abstract:** The protein deacetylase sirtuin 1 (SIRT1) is an established regulator of diverse physiological processes and one of several promising targets for pharmacologic modulation of ageing and longevity. In normal human keratinocytes, SIRT1 has been shown to inhibit proliferation and promote differentiation. MicroRNAs (miRNAs), small non-coding RNA molecules that negatively regulate gene expression, have been shown to control SIRT1 expression in several cell types. Using western blotting, we show that miR-9 represses SIRT1 expression in HaCaT human keratinocytes. The attenuation of SIRT1 levels in response to ectopic miR-9 occurred in a dose-dependent manner. As miR-9 expression is known to be under epigenetic control, the effect of the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) was examined. Levels of mature miR-9 increased 8-fold following TSA treatment of HaCaT keratinocytes. Expression of the primary transcripts from which miR-9 is derived was also raised in HaCaT keratinocytes exposed to TSA, with a 7-fold elevation of pri-miR-9-1 and 4-fold increase of pri-miR-3. In contrast the DNA methyl transferase inhibitor 5-deoxy-azacytidine (DAC) had little effect on miR-9 or primary miR-9 expression. Together, our findings point to a role for chromatin remodelling in regulating miR-9 levels in human keratinocytes and in turn modulation of SIRT1 expression by miR-9.

**Keywords:** microRNA; keratinocytes; sirtuin 1 ;trichostatin A.

### INTRODUCTION

Sirtuins (SIRT1 to 7) are a family of conserved protein deacetylases that regulate gene expression in response to the metabolic status of cells. Intracellular levels of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) control SIRT1 activity and SIRT1-dependent deacetylation of histone lysines allows chromatin remodelling to support the regulation of genes involved in a multiple physiological processes[1]. Activation of SIRT1 has been linked to the extension of lifespan in multiple animal models and is therefore a promising pharmaceutical target for healthy ageing[2].

The exposure of the skin to ultraviolet (UV) radiation is associated with photoageing and the development of skin cancer [3,4]. Recent insight from mouse studies suggest gene dosage determines SIRT1 function in UVB-induced skin cancer, with enhanced tumourigenesis following heterozygous deletion but a tumour suppressive effect for homozygous deletion SIRT1 [5]. In addition, upregulation of SIRT1 has been shown to promote differentiation and shorten the replicative lifespan of cultured keratinocytes [6]. Conversely, depletion of SIRT1 inhibited differentiation and enhanced the replicative capacity of keratinocytes [6]. However, the mechanisms regulating SIRT1 expression in keratinocytes have not been fully established.

MicroRNAs (miRNAs) are small (~22 nucleotides) non-coding RNA molecules that control gene expression, generally by post-transcriptional down regulation of target mRNA at the protein and/or mRNA level[7,8]. A given miRNA can regulate hundreds of distinct mRNA transcripts and consequently, changes in miRNA expression can significantly influence physiological and pathological processes [9]. Mature miRNAs are generated from primary transcripts (pri-miRNAs) which are usually transcribed from intergenic or intronic regions of protein-coding genes [10]. The RNase III enzyme Drosha processes the primary transcripts into precursor miRNAs (pre-miRNAs) which are in turn cleaved by another RNase III, Dicer, to produce the mature miRNA[11].

Increasing evidence points the regulation of SIRT1 by miR-9 in diverse cell types [12-14]. In the study presented here, we extend the known cell types in which miR-9 modulates SIRT1 expression to include HaCaT keratinocytes. Furthermore, we show that treatment of HaCaT keratinocytes with the histone deacetylase inhibitor trichostatin A (TSA) leads to the upregulation of mature and primary miR-9.

## MATERIALS AND METHODS

### Transient transfection and Western Blotting

The p-Super-miR-9 and pSuper vectors [15] were kind gifts from Romano Regazzi (University of Lausanne, Switzerland). Cells seeded at  $3 \times 10^5$  cells/well of a six well plate were transfected using Lipofectamine Plus reagents (Thermo Fisher, Paisley, UK) according to manufacturer's instructions. After 48 h, the cells were lysed by replacing the culture medium with 250µl urea lysis buffer (0.1M Tris-Cl pH 6.8, 7M urea, 4% SDS, protease inhibitor cocktail). For western blotting, 20µl of protein lysate per lane of a 4-12% gradient polyacrylamide gel (Expedeon, Harston, UK) was fractionated by electrophoresis, transferred to a polyvinylidene fluoride membrane, and probed using anti-human SIRT1 antibody (1:1000; Abcam Cambridge, UK) or  $\beta$ -actin (1:2000; Abcam). After incubation with horseradish peroxidase-conjugated secondary antibody (1:10,000, Abcam), peroxidase-dependent chemiluminescence was visualized using Pierce reagents (Thermo Scientific, Rockford, IL, USA). Densitometry was performed using Image J software and values for SIRT1 normalized to the corresponding  $\beta$ -actin values.

### RNA isolation and purification

HaCaT keratinocytes were cultured as previously reported [16]. Cells were treated with 1 µM trichostatin A (TSA) or 5-deoxy-azacytidine (DAC), respectively, and harvested 48 h later. Cell lysis and total RNA purification was performed using the Ambion miRVANA (Applied Biosystems) RNA extraction kit in accordance with the manufacturer's specifications. Briefly, cells were washed 3 times in PBS prior to addition of 600µl miRVANA lysis buffer (Ambion). The lysate was subsequently transferred to a tube containing 60µl miRNA homogenate additive and mixed by vortexing. After a 10 min incubation on ice, 500µl of acid-phenol:chloroform was added to the lysate. The mixture was vortexed for 30 s and centrifuged ( $10,000 \times g$  for 5 min) to separate the organic and aqueous phases. The aqueous phase was transferred to a microcentrifuge tube, mixed with 100% ethanol and passed through a glass fibre filter by centrifugation ( $10,000 \times g$  for 15 s). The filter was washed and total RNA was recovered with pre-heated ( $95^\circ\text{C}$ ) elution solution.

### Quantitative RT-PCR

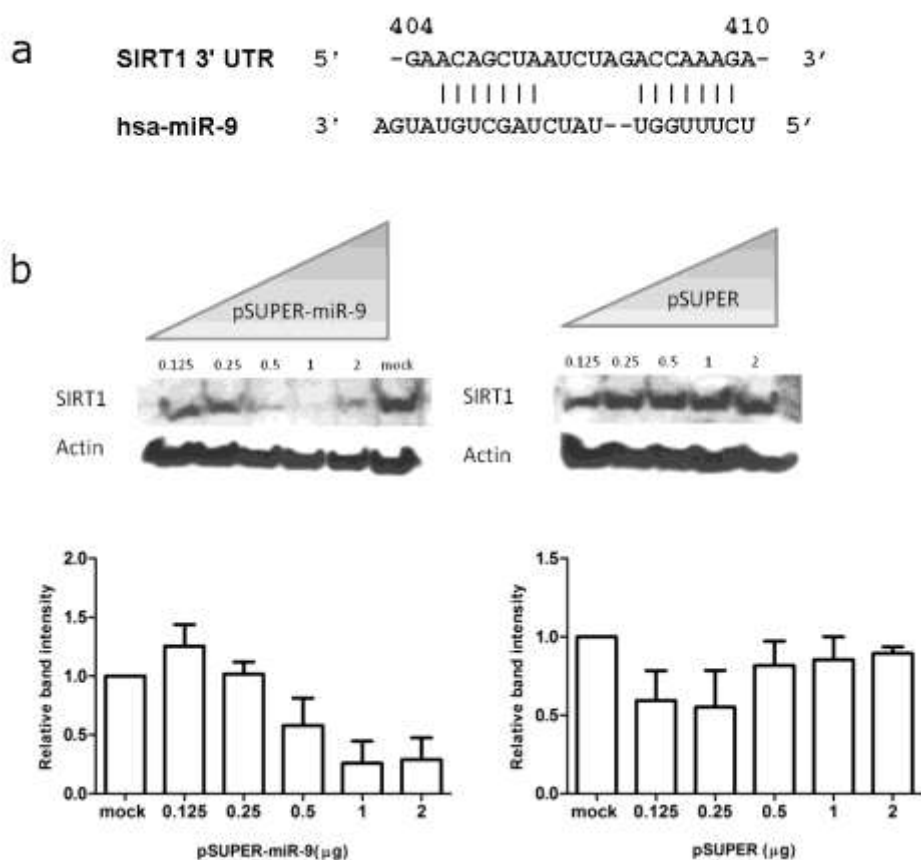
Quantitative PCR (qRT-PCR) for miR-9 expression was performed using a TaqMan miRNA assay (Applied Biosystems). Briefly, 20 ng of total RNA was reverse transcribed according to manufacturer's instructions using a stem loop primer specific for miR-9 or for the endogenous control gene RNU48, respectively. Quadruplicate qPCR mixtures were then prepared, each 20µl reaction comprising 10µl TaqMan Universal PCR Master Mix II (2x; Applied Biosystems), 7.67µl nuclease free H<sub>2</sub>O (Sigma Aldrich), 1µl TaqMan primers (20x) for miR-9 or RNU48 quantification, respectively (Applied Biosystems), and 1.33µl of RT product. Thermocycling was performed on an AB 7500 real time PCR system (Applied Biosystems) as follows: incubation at  $50^\circ\text{C}$  for 2 min, incubation at  $95^\circ\text{C}$  for 10 min, then 40 cycles of  $95^\circ\text{C}$  for 15 s and  $60^\circ\text{C}$  for 1 min.

For quantification of pri-miR-9-1 and pri-miR-9-3, 200 ng of total RNA was reverse transcribed and target expression assessed using TaqMan pri-miRNA assays (Applied Biosystems) for pri-miR-9-1, pri-miR-9-3, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels were also determined as an endogenous control. Preparation of qPCR mixtures and thermocycling were performed as outlined above.

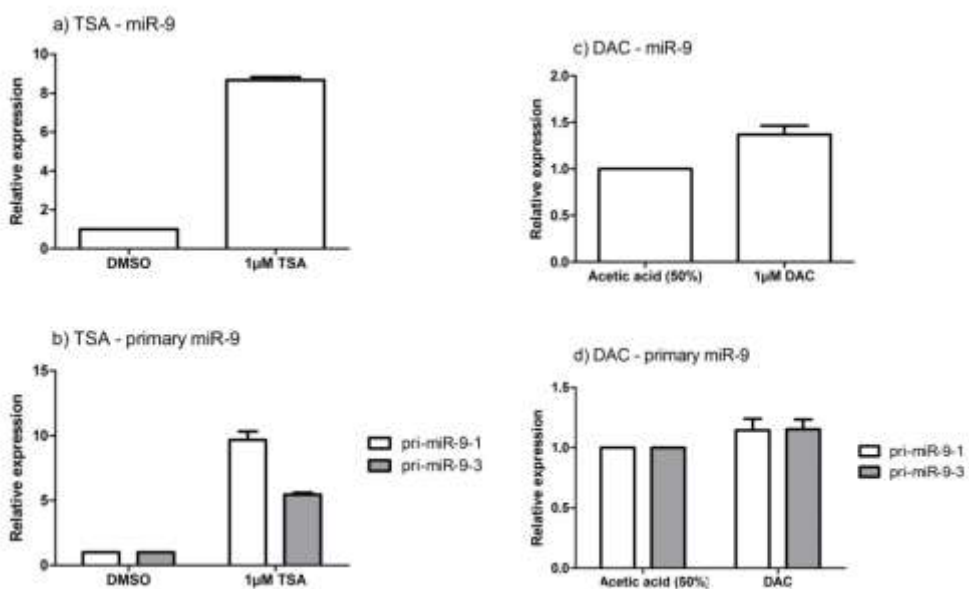
Relative gene expression was determined using the comparative cycle threshold ( $C_T$ ) method [17]. Values for miR-9 were normalized to those of RNU48; values for pri-miR-9-1 and pri-miR-9-3 to GAPDH mRNA levels, respectively.

## RESULTS

In HaCaT keratinocytes transfected with a miR-9 expression plasmid, a clear dose-dependent attenuation of SIRT1 levels was observed as the plasmid doses exceeded 0.25 µg (Fig.1). The lowest dose (0.125 µg) of the miR-9 plasmid, however, appeared to potentiate SIRT1 expression. No dose-dependent depletion of SIRT1 was observed in cells transfected with an empty control plasmid.



**Fig-1: miR-9 as a regulator of SIRT1 protein expression in keratinocytes.** The interaction of miR-9 with its predicted target site in human SIRT1 3'UTR is depicted (a). HaCaT keratinocytes were transfected with increasing amounts ( $\mu\text{g}$ ) of vector encoding miR-9 or pSuper empty vector (b). Relative SIRT1 levels (means  $\pm$  SEM,  $n = 3$ ), normalized to  $\beta$ -actin, are presented below each panel.



**Fig-2: Regulation of miR-9 expression in HaCaT keratinocytes.** Cells were treated with the indicated doses of TSA, DAC or vehicle for 48 h. Data shown represents the mean values obtained from 3 independent experiments.

Given that miR-9 was reported to be silenced epigenetically in cancer cell lines [18], we evaluated the effects of the histone deacetylase inhibitor TSA and the DNA methyl transferase (DNMT) inhibitor DAC on miR-9 expression. As shown in Fig, 2a, TSA treatment resulted in an 8-fold increase in miR-9 expression after 48 h. To confirm whether the elevation of mature miR-9 was associated with upregulation of its primary transcripts, the expression of two pri-miR-9 transcripts were also examined. The levels of pri-miR-9-1 and pri-miR-9-3 were raised 7-fold and 4-fold, respectively, by TSA treatment (Fig.2b). In contrast, exposure to DAC has little effect of the expression of mature or primary miR-9 (Fig.2c,d). Hence epigenetic silencing of miR-9 in keratinocytes appears to be associated with HDAC rather than DNMT activity.

## DISCUSSION

SIRT1 is a protein deacetylase that has been implicated in the regulation of cellular and organismal ageing [9]. Recent work indicates that miR-9 regulates SIRT1 in mouse embryonic stem cells [12] and pancreatic  $\beta$ -islet cells[13]. In keratinocytes, depletion of SIRT1 appears to prolong replicative capacity, indicating that SIRT1 may contribute to the regulation of keratinocyte proliferation [6]. In the study presented here, we have shown that ectopic expression of miR-9 in human epidermal keratinocytes downregulates SIRT1. Thus miR-9 joins several miRNAs, including miR-138, miR-181a and miR-181b, which have been shown to regulate SIRT1 expression in human keratinocytes [19]. However, miR-138, miR-181a and miR-181b were induced during keratinocyte senescence, whereas miR-9 was not reported as a senescence-associated miRNA in keratinocytes. Thus the function of the miR-9:SIRT1 axis in keratinocytes remains to be fully defined.

Elevation of global histone acetylation by HDAC inhibition with TSA is known to modulate keratinocyte differentiation[20-22]. As we observed TSA-dependent induction of miR-9 in HaCaT keratinocytes, chromatin remodelling may contribute to the elevation of miR-9 that has been reported in terminally differentiated keratinocytes [23]. In contrast to findings from cancer cells[18], we did not observe de-repression of miR-9 following treatment of keratinocytes with DAC suggesting diverse epigenetic mechanisms control miR-9 expression in different cell types. These mechanisms may be augmented by other pathways known to contribute to the expression of miR-9, such as Toll-like receptor, IL-1 $\beta$  and TNF- $\alpha$  signalling [24].

In conclusion, we have shown in this study that upregulation of miR-9 leads to repression of SIRT1 and the histone deacetylase inhibitor TSA elevates miR-9 expression in human keratinocytes.

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## REFERENCES

1. Kupis, W., Palyga, J., Tomal, E., and Niewiadomska, E. (2016) The role of sirtuins in cellular homeostasis. *J Physiol Biochem* 72, 371-380
2. Longo, V. D., Antebi, A., Bartke, A., Barzilai, N., Brown-Borg, H. M., Caruso, C., Curiel, T. J., de Cabo, R., Franceschi, C., Gems, D., Ingram, D. K., Johnson, T. E., Kennedy, B. K., Kenyon, C., Klein, S., Kopchick, J. J., Lepperdinger, G., Madeo, F., Mirisola, M. G., Mitchell, J. R., Passarino, G., Rudolph, K. L., Sedivy, J. M., Shadel, G. S., Sinclair, D. A., Spindler, S. R., Suh, Y., Vijg, J., Vinciguerra, M., and Fontana, L. (2015) Interventions to Slow Aging in Humans: Are We Ready? *Aging cell*, 14, 497-510
3. Narayanan, D. L., Saladi, R. N., and Fox, J. L. (2010) Ultraviolet radiation and skin cancer. *Int J Dermatol*, 49, 978-986
4. Yaar, M., and Gilchrist, B. A. (2007) Photoageing: mechanism, prevention and therapy. *Br J Dermatol*, 157, 874-887
5. Ming, M., Soltani, K., Shea, C. R., Li, X., and He, Y. Y. (2015) Dual role of SIRT1 in UVB-induced skin tumorigenesis. *Oncogene* 34, 357-363
6. Blander, G., Bhimavarapu, A., Mammone, T., Maes, D., Elliston, K., Reich, C., Matsui, M. S., Guarente, L., and Loureiro, J. J. (2009) SIRT1 Promotes Differentiation of Normal Human Keratinocytes. *J Invest Dermatol*, 129, 41-49;
7. Wilczynska, A., and Bushell, M. (2015) The complexity of miRNA-mediated repression. *Cell Death Differ*, 22, 22-33
8. Jonas, S., and Izaurralde, E. (2015) Towards a molecular understanding of microRNA-mediated gene silencing. *Nat Rev Genet*, 16, 421-433
9. Sayed, D., and Abdellatif, M. (2011) MicroRNAs in development and disease. *Physiol Rev*, 91, 827-887
10. Kim, V. N., Han, J., and Siomi, M. C. (2009) Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* 10, 126-139
11. Winter, J., Jung, S., Keller, S., Gregory, R. I., and Diederichs, S. (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol*, 11, 228-234
12. Saunders, L. R., Sharma, A. D., Tawney, J., Nakagawa, M., Okita, K., Yamanaka, S., Willenbring, H., and Verdin, E. (2010) miRNAs regulate SIRT1 expression during mouse embryonic stem cell differentiation and in adult mouse tissues. *Aging (Albany NY)*, 2, 415-431
13. Ramachandran, D., Roy, U., Garg, S., Ghosh, S., Pathak, S., and Kolthur-Seetharam, U. (2011) Sirt1 and mir-9 expression is regulated during glucose-

- stimulated insulin secretion in pancreatic beta-islets. *FEBS J*, 278, 1167-1174
14. Schonrock, N., Humphreys, D. T., Preiss, T., and Gotz, J. (2012) Target gene repression mediated by miRNAs miR-181c and miR-9 both of which are down-regulated by amyloid-beta. *J Mol Neurosci* 46, 324-335
  15. Plaisance, V., Abderrahmani, A., Perret-Menoud, V., Jacquemin, P., Lemaigre, F., and Regazzi, R. (2006) MicroRNA-9 controls the expression of Granuphilin/Slp4 and the secretory response of insulin-producing cells. *J Biol Chem*, 281, 26932-26942
  16. Roberts, J. C., Warren, R. B., Griffiths, C. E., and Ross, K. (2013) Expression of microRNA-184 in keratinocytes represses argonaute 2. *J Cell Physiol* 228, 2314-2323
  17. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>(Delta Delta C(T)) Method. *Methods*, 25, 402-408
  18. Lujambio, A., Calin, G. A., Villanueva, A., Ropero, S., Sanchez-Cespedes, M., Blanco, D., Montuenga, L. M., Rossi, S., Nicoloso, M. S., Faller, W. J., Gallagher, W. M., Eccles, S. A., Croce, C. M., and Esteller, M. (2008) A microRNA DNA methylation signature for human cancer metastasis. *Proc Natl Acad Sci U S A* 105, 13556-13561
  19. Rivetti di Val Cervo, P., Lena, A. M., Nicoloso, M., Rossi, S., Mancini, M., Zhou, H., Saintigny, G., Dellambra, E., Odorisio, T., Mahe, C., Calin, G. A., Candi, E., and Melino, G. (2012) p63-microRNA feedback in keratinocyte senescence. *Proc Natl Acad Sci U S A*, 109, 1133-1138
  20. Saunders, N., Dicker, A., Popa, C., Jones, S., and Dahler, A. (1999) Histone deacetylase inhibitors as potential anti-skin cancer agents. *Cancer Res*, 59, 399-404
  21. Connelly, J. T., Mishra, A., Gautrot, J. E., and Watt, F. M. (2011) Shape-induced terminal differentiation of human epidermal stem cells requires p38 and is regulated by histone acetylation. *PLoS One*, 6, e27259
  22. Markova, N. G., Karaman-Jurukovska, N., Pinkas-Sarafova, A., Marekov, L. N., and Simon, M. (2007) Inhibition of histone deacetylation promotes abnormal epidermal differentiation and specifically suppresses the expression of the late differentiation marker profilaggrin. *J Invest Dermatol*, 127, 1126-1139
  23. Hildebrand, J., Grundhoff, A., Gallinat, S., Wenck, H., and Knott, A. (2013) MicroRNA profiling during human keratinocyte differentiation using a quantitative real-time PCR method. *Methods Mol Biol*, 961, 193-200
  24. Bazzoni, F., Rossato, M., Fabbri, M., Gaudiosi, D., Mirolo, M., Mori, L., Tamassia, N., Mantovani, A., Cassatella, M. A., and Locati, M. (2009) Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proc Natl Acad Sci U S A*, 106, 5282-5287