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Dysregulation of microRNA expression in diabetic skin

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ABSTRACT

Background: Clinical skin manifestations are common in diabetes; however, molecular mechanisms underlying such defects are largely unknown. Several findings indicate a role for microRNAs (miRNAs) in skin homeostasis.

Objective: To investigate whether miRNA expression is altered in diabetic skin.

Methods: Type 1 and 2 mouse models of diabetes were used. MiRNA profiling was performed on RNA extracted from the skin of type 1 diabetic mice and non-diabetic controls. Expression levels of pri-miRNAs and of miRNA-biogenesis genes were also analyzed. Biogenesis gene expression analysis was performed in human dermal fibroblasts cultured in hyperglycemic, hypoxic or oxidative stress conditions.

Results: Several miRNAs were differentially expressed in diabetic skin with a general down-modulation as compared to controls. Bioinformatics analysis of signature-miRNA target genes showed the enrichment in pathways involved in skin homeostasis, such as TGF- β and Wnt. MiRNA alteration in diabetic skin associated with reduced expression levels of DROSHA, DGCR8, XPO5, DICER1, AGO2, both as mRNA and protein. Reduced biogenesis gene expression did not correlate with accumulation of pri-miRNAs, which displayed differences in expression levels similar to those found for their mature miRNAs. Experiments with cultured fibroblasts showed that hypoxia and oxidative stress induced the down-regulation of miRNA-biogenesis genes in this skin cell type.

Conclusion: A general down-regulation of differentially expressed miRNAs was found in diabetic skin. This alteration is part of and is dependent from a wider transcriptional defect also affecting the expression of pri-miRNAs and of genes responsible for miRNA biogenesis. Such an alteration is likely contributing to diabetic skin manifestations.

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1. Introduction

Diabetes is a chronic metabolic disease whose incidence and prevalence are high and constantly growing [1]. It arises as consequence of increased glucose levels due to insulin unbalance or impaired responsiveness. The vast majority of diabetic patients suffers from type 2 diabetes due to insulin resistance, while 5–10% of patients are affected by type 1 insulin-dependent diabetes [1]. Clinical skin manifestations are common in diabetes, often being the first sign of the metabolic disorder [2]. They involve at least 30% of patients and include increased propensity to infections, hyperpigmentation, dermal thickening, spontaneous blister development and the highly invalidating foot ulcers. Skin

complications are due to metabolic and physiological consequences typical of the diabetic condition. Impaired cell activity resulting from hyperglycemia, persistent inflammation and increased oxidative stress plays major roles in the healing defect in diabetic foot ulcer [3]. Glycation affects protein functions and strongly contributes to impaired cell activity in diabetes [4]. Altered gene expression also plays a role in diabetic dysfunctions; dysregulated gene expression was found in diabetic skin, with an increase in gene pathways involved in immune response and infectious diseases [5]. Moreover, differences in gene expression were associated with reduced biomechanical properties of diabetic skin [6]. A better understanding of the molecular alterations underlying skin defects in the diabetic condition may help in better managing diabetes-associated skin complications.

MicroRNA (miRNA) are small, non-coding RNAs regulating gene expression at post-transcriptional level. By binding to mRNA sequences in the 3' untranslated region of target mRNAs, they

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primarily downregulate gene expression, thus playing a major role in all main biological processes, including skin development and homeostasis [7]. miRNA dysregulation contributes to the onset and development of human disorders. As for diabetes, experimental findings indicate that miRNA altered expression is involved in diabetic complications, including the healing impairment of diabetic skin [8,9].

To further investigate whether miRNA dysregulation plays a role in diabetic skin alterations, we compared miRNA baseline expression levels in diabetic and non-diabetic skin in a mouse model of type 1 diabetes by performing a wide miRNome analysis. Data were confirmed on type 2 diabetic mice. Having found miRNA widespread decrease in diabetic skin, the expression of genes involved in the miRNA biogenesis process as well as pri-miRNA levels were analyzed. The effect of hypoxia, hyperglycemia or oxidative stress in regulating miRNA-biogenesis genes in the skin context was also investigated in cultured dermal fibroblasts.

2. Materials and methods

2.1. Diabetic mice

Mice were purchased from Charles River Laboratories (Calco, Lecco, Italy). To obtain type 1 diabetic mice, six weeks-old C57Bl/6 male mice were intraperitoneally injected with 160 mg/kg

streptozotocin (Sigma-Aldrich, St. Louis, MO) in 0.05 M Na citrate pH 4.5. Seven days after, blood glucose levels were measured, and mice with glycemia above 250 mg/dL were included in the study. Three weeks later, glycemia was checked again, diabetic mice sacrificed and skin collected.

Ten weeks-old C57Bl/KS-Lepr db/db male mice, homozygous for the spontaneous mutation of the leptin receptor (*Lepr*), and heterozygote littermates (db/+) were purchased. After one week, glycemia was checked, mice sacrificed and skin collected. Db/db mice displayed glycemia above 500 mg/dL; db/+ mice had a glycemia of 139 ± 27.7 mg/dL.

Animal procedures were approved by the Animal Committee of the Italian Ministry of Health (approvals n. SA-IDI-12-TO-1 and n. 1016/2016-PR).

2.2. Mouse samples

Back skin biopsies were snap-frozen or fixed in 10 % formalin and paraffin-embedded. Frozen biopsies were crushed to powder that was poured into TRIzol (Termofisher Scientific, Waltham, MA, USA) for total RNA extractions. For protein extraction, the frozen powder was dissolved in RIPA buffer containing protease inhibitors; lysates were incubated in ice for 15 min and centrifuged at 16,000 g for 15 min. Supernatants were frozen to -80 °C.

Table 1

Pathways with significant enrichment of experimentally validated target genes of miRNAs down-modulated in the skin of STZ diabetic mice. KEGG pathway analysis of target genes of down-regulated miRNAs show pathways with involvement in neuronal function or playing an important role in the skin, such as TGF- β and Wnt (#5 and #10, respectively). HIF-1 and VEGF signaling were also found among the significantly regulated pathways (#29 and #35, respectively).

#	KEGG pathway	n. genes	n. miRNAs	p-value
1	Lysine degradation	8	2	6.93×10^{-11}
2	Axon guidance	16	2	3.31×10^{-8}
3	Gap junction	8	3	5.72×10^{-7}
4	Retrograde endocannabinoid signaling	12	2	1.30×10^{-6}
5	TGF- β signaling	9	4	3.49×10^{-6}
6	Long-term potentiation	9	4	4.29×10^{-6}
7	Gastric acid secretion	9	4	2.17×10^{-5}
8	Gabaergic synapse	9	2	0.00017
9	GnRH signaling pathway	9	4	0.00018
10	Wnt signaling pathway	14	5	0.00021
11	Glutamatergic synapse	11	2	0.00048
12	Dorso-ventral axis formation	4	3	0.00048
13	ErbB signaling pathway	9	5	0.00064
14	Dopaminergic synapse	11	4	0.00130
15	Amphetamine addiction	7	2	0.00133
16	Chagas disease	9	2	0.00289
17	Circadian entrainment	9	4	0.00330
18	Adherens junction	8	2	0.00355
19	Cholinergic synapse	9	4	0.00457
20	Nicotine addiction	5	2	0.00565
21	Colorectal cancer	6	3	0.00565
22	Long-term depression	5	2	0.01398
23	Tuberculosis	10	4	0.01631
24	Aldosterone-regulated sodium reabsorption	4	2	0.02006
25	Salivary secretion	6	2	0.02006
26	Alzheimer's disease	11	2	0.02006
27	Melanogenesis	7	4	0.02006
28	Amiotrophic lateral sclerosis	5	2	0.02024
29	HIF-1 signaling pathway	8	2	0.02076
30	Pancreatic secretion	6	2	0.02146
31	Salmonella infection	6	2	0.02146
32	B cell receptor signaling pathway	6	2	0.02305
33	Hepatitis B	8	3	0.02683
34	Fatty acid elongation	2	1	0.03625
35	VEGF signaling pathway	5	2	0.03625
36	Serotonergic synapse	8	2	0.03625
37	NOD-like receptor signaling pathway	5	1	0.03861
38	Morphine addiction	7	2	0.03896
39	MAPK signaling pathway	14	4	0.03896
40	Progesterone-mediated oocyte maturation	6	2	0.03930
41	Spliceosome	9	2	0.04113

2.3. MiRNA array

Two-color hybridization was performed with miRCURY LNA microRNA arrays (7th generation [miRBase v18]; Exiqon, Vedbæk, Denmark), co-hybridizing total RNA from each type 1 diabetic mouse ($n = 6$) with total RNAs from a pool of 6 non-diabetic animals.

1.25 μ g of total RNA was labeled with either Hy3 (non-diabetic pool) or Hy5 (diabetic) using the miRCURY LNA microRNA Array Power labeling kit (Exiqon), according to the manufacturer's protocol. Additionally, two dye-swaps were performed, labeling the diabetic sample with Hy3 and the control pool with Hy5.

Hybridization at 53 °C for 16 h and washing steps were carried out on the HS 400 PRO hybridization station (Tecan, Männedorf, Switzerland). MicroRNA arrays were scanned using the GenePix 4100A microarray scanner and the GenePix Pro GenePix Pro 6.1.0.4 software (Molecular Devices, San Jose, CA, USA).

2.4. Global miRNA analysis

Signal intensities were analyzed with the limma package (Bioconductor 2.5 on R 2.10.1) [10,11]. Background correction (normexp; cut-off = 10) and within-array normalization (global LOESS) was performed. Differential expression and statistical significance were assessed for each miRNA species across all arrays using linear model fit and empirical Bayes method (lmFit, eBayes), taking into account the dye-swap. Spots showing intensities < 3 SNR (for Hy3 and Hy5) OR displaying diameters >130 or <70 μ m were considered as bad quality spots. The calling of a miRNA failed if out of its 4 replicates ≥ 2 were bad quality spots and if this occurred on ≥ 4 arrays. Normalized log₂ ratios represent diabetic/non-diabetic (Hy5/Hy3). *P*-values are adjusted for False Discovery Rate (Benjamini and Hochberg's method) [12]. Data are available on the NCBI Gene Expression Omnibus data base, GEO series GSE137605.

2.5. MiRNA pathway analysis

The list of differentially expressed miRNAs was filtered for significance using adjusted *p*-value of ≤ 0.10 as threshold (Supplementary Table 1). The resulting list was separated by modulation, and down-modulated miRNAs were used for KEGG-pathway analysis by DIANA miRPath v.2.0, selecting the experimentally validated miRNA interactions comprised in DIANA-TarBase [13].

2.6. Real time RT-PCR

Ten ng of total RNA were converted into cDNA with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), and templates were amplified in real time RT-PCR by using Premix Ex Taq (Probe qPCR) (Takara Bio, Shiga, Japan) and specific TaqMan MicroRNA Assays (Applied Biosystems). Otherwise, 5 μ g of RNA were retrotranscribed with M-MLV reverse transcriptase. The cDNAs were amplified with Premix Ex Taq (Probe qPCR) (Takara Bio) and specific TaqMan Pri-miRNA Assays (Applied Biosystems), or with the SYBR Premix Ex Taq II (Takara Bio) and primers for mRNA sequences (Supplementary Table 2). TATA-box binding protein (*TBP*) was identified as most suitable reference gene for data normalization of both mRNAs and pri-miRNAs after having tested its mRNA levels and those of other five housekeeping genes in diabetic and non-diabetic samples. Candidate reference genes were ranked according to their expression stability by the geNorm application for Microsoft Excel (data not shown) [14]. MiR-16 displayed a similar expression in

diabetic and non-diabetic samples (logFC -0.16 , adj *p*-value 0.86), and was selected for miRNA normalization.

For PCR detected with the SYBR-Green dye, the relative expression ratio of target genes was computed with the Light-Cycler 2.0 Relative Quantification software (Roche Diagnostics, Basel, Switzerland), by applying the correction for PCR efficiency. For PCR performed with TaqMan probes, calculation of data was executed by means of the $\Delta\Delta$ CT method.

2.7. Western blot

Fifty μ g of tissue extracts were electrophoresed on polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were saturated with 5% low fat milk in TBST (50 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20, pH 7.5) for 2 h at room temperature. Incubations with primary antibodies were performed in TBST with 2.5% low fat milk at 4 °C overnight. Antibodies were diluted as follows: Anti-XPO5 (ab131281, Abcam, Cambridge, UK) 1:3,000; anti-DGCR8 (ab191875, Abcam) 1:3000; anti-AGO2 (ab57113, Abcam) 1:1000; anti-DROSHA (ab12286, Abcam) 1:1000; anti-DICER1 (sc-30226, Santa Cruz Biotechnology, Dallas, TX, USA) 1:1000; anti- β -tubulin (T2200, Sigma-Aldrich) 1:20,000; anti-vinculin (ab129002, Abcam) 1:20,000. Horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Cambridgeshire, UK) were used in TBST with 2.5% low fat milk for 1 h. Western blots were revealed by chemiluminescence.

2.8. Immunofluorescence

Four-micrometer-sections of paraffin-embedded skin specimens were rehydrated and antigen retrieved prior staining with the anti-DICER1 antibody (sc-30226, Santa Cruz Biotechnology) diluted 1:25. Sections were analyzed with Axioplan2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with an Axiocam camera. Staining was quantified with ImageJ (imagej.nih.gov/ij/index.html) after applying a constant threshold.

2.9. Culture conditions

Human dermal primary fibroblasts isolated from foreskin samples were cultured in F10 medium (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 0.5% penicillin and 2% glutamine.

For hypoxic conditions, fibroblasts were maintained for 24 h (for RNA analysis) or 48 h (for protein analysis) in 1% O₂ by using a Hypoxia Incubator Chamber (Stemcell Technologies, Grenoble, France).

The oxidative stress was produced by adding hydrogen peroxide (H₂O₂) to cultured fibroblasts at different doses (0–200 μ M) for the indicated times. Cell proliferation was measured by colorimetric immunoassay based on BrdU incorporation (Roche Diagnostics). Total cellular glutathione was detected after 4 h incubation by means of the GSH + GSSG/GSH Assay Kit (Abcam).

Chronic hyperglycemic conditions were obtained by culturing fibroblasts for three weeks in commercially available Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) containing 25 mM or 5.5 mM D-glucose. The high glucose and low glucose DMEM had similar osmolarity.

2.10. Statistical analysis

The test U of Mann-Whitney was used to compare pri-miRNAs, miRNAs, mRNAs and protein levels in control and diabetic mice, while *t*-test was used for the *in vitro* experiments with cultured fibroblasts.

3. Results

3.1. MiRNA profiling reveals a miRNA general down-modulation in the skin of diabetic mice

MiRNA profiling was performed by miRNome analysis on RNA extracted from the skin of streptozotocin-induced diabetic mice, a model of type 1 diabetes, and non-diabetic mice ($n = 6/\text{group}$). More than 400 different miRNA species were identified (GSE137605). Of these, 30 were significantly modulated, showing an FDR adjusted p -value ≤ 0.10 . Strikingly, among these 30, 27 were down-regulated and only 3 were up-regulated in diabetic mice (Fig. 1 A, Supplementary Table 1). These findings were validated by real-time RT-PCR in skin samples from a different group of diabetic mice and non-diabetic controls ($n = 8/\text{group}$) by analyzing a subset of miRNAs (Fig. 1B). MiRNAs selected for validation were those found to be more down-regulated or having validated target genes (miR-541-5p, miR-136-5p, and miR-148a-3p). MiR-139-5p, one of the up-regulated miRNAs, was also analyzed for validation. All miRNAs examined were confirmed to be expressed at significantly different levels. The same miRNAs were analyzed in the skin of the db/db mice, a model of type 2 diabetes, compared to db/+ non-diabetic mice ($n = 6/\text{group}$). Differences in expression levels for selected miRNAs were found also in type 2 diabetic mice (Fig. 1 C).

KEGG pathway analysis on down-regulated miRNAs with validated targets was performed (Table 1). Among the most involved pathways there were TGF- β and Wnt signaling, both

with proved roles in skin homeostasis, in wound healing and in diabetic complications [15–17]. Moreover, a number of target genes were involved in neuronal development and neurotransmission pathways. Hypoxia inducible factor-1 α (HIF-1 α) and vascular endothelial growth factor (VEGF) signaling pathways, important for skin angiogenesis and in cutaneous wound healing [18–21], were also among the pathways significantly involved.

3.2. Genes responsible for miRNA biogenesis are down-modulated in the skin of diabetic mice

The higher number of down-modulated miRNAs in the skin of diabetic mice may reflect either a reduced transcription rate or an impairment of miRNA biogenesis in the diabetic condition. We therefore investigated whether the expression of genes involved in the canonical miRNA biogenesis pathway was altered in the diabetic skin. The following genes were analyzed: the ribonuclease III *DROSHA* and Di George Syndrome Critical Region 8 (*DCGR8*), both mediating primary miRNA (pri-miR) to precursor-miRNA (pre-miR) maturation within the nucleus; Exportin 5 (*XPO5*), allowing pre-miR exportation to the cytoplasm; the ribonuclease III *DICER1* and the Argonaute member 2 (*AGO2*) of the miRNA-induced silencing complex (miRISC), responsible for miRNA terminal maturation in the cytoplasm [22]. All genes displayed significantly lower mRNA levels in the skin of streptozotocin-induced diabetic mice ($n = 8$) and db/db mice ($n = 6$) as compared to non-diabetic controls (Fig. 2A and B).

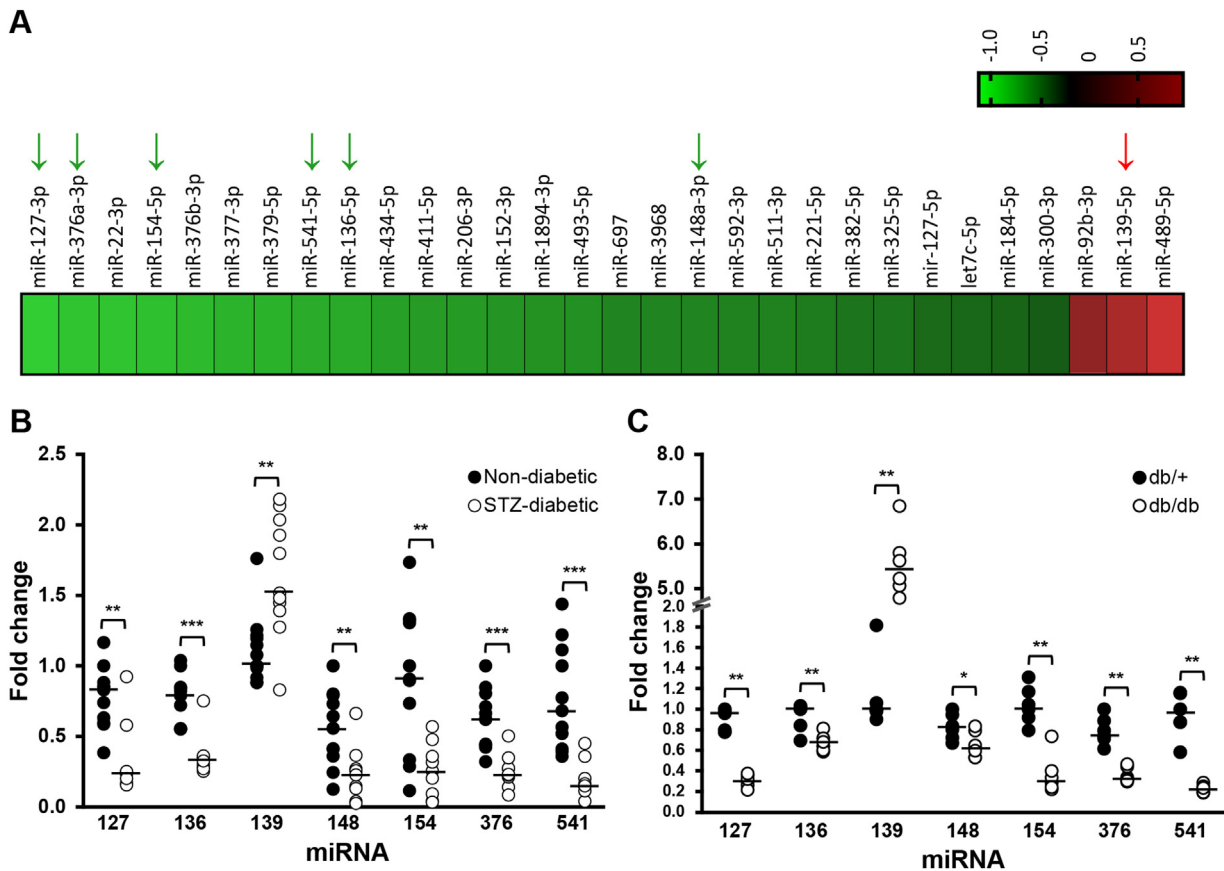


Fig. 1. Comparison of miRNA expression in the skin of diabetic and non-diabetic mice. (A) Heat map of miRNAs expressed at different levels in the skin of streptozotocin-induced diabetic mice as compared to non-diabetic controls ($n = 6/\text{group}$) sorted by increasing logFC. Out of 30 miRNAs, only 3 were upregulated. Arrows mark miRNAs selected for validation. (B) Validation by real-time RT-PCR of the differences observed in the expression of selected miRNAs in the skin of a different group of streptozotocin-induced diabetic mice (STZ) and non-diabetic mice ($n = 8/\text{group}$). miR16 was used as reporter gene. (C) Validation by real-time RT-PCR of the differences observed in the expression of selected miRNAs in the skin of db/db mice and db/+ control mice ($n = 6/\text{group}$) (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

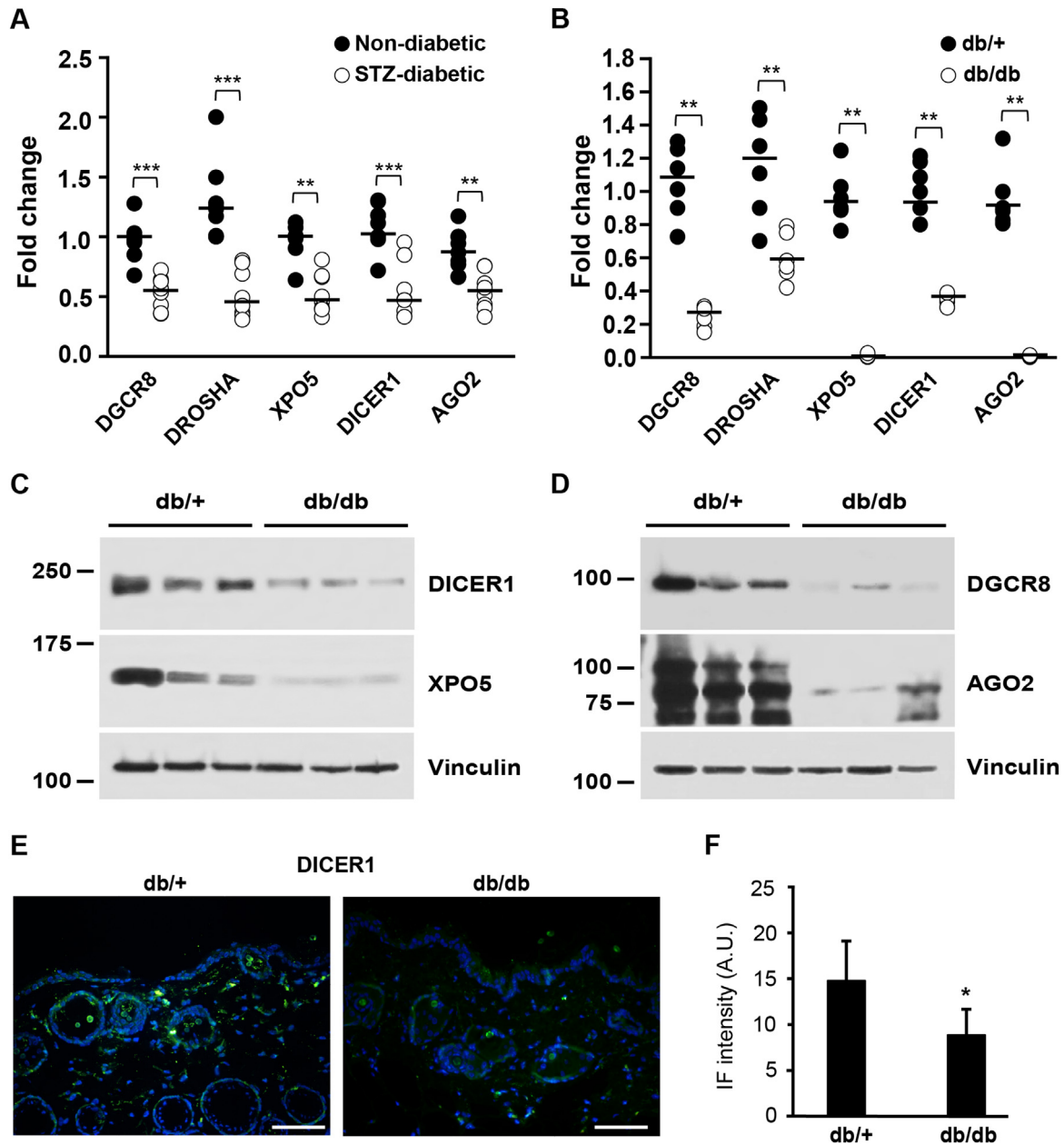


Fig. 2. Reduced expression levels of genes involved in miRNA biogenesis in the skin of diabetic mice. (A) Real-time RT-PCR for genes involved in miRNA maturation performed in skin mRNA samples of streptozotocin-induced (STZ) diabetic mice and non-diabetic controls ($n = 8$ /group). TATA-box binding protein (TBP) was used as reporter gene. (B) Real-time RT-PCR for the same genes in skin mRNA samples of db/db diabetic mice and db/+ non-diabetic controls ($n = 6$ /group). (C,D) Western blot for DICER1 and XPO5 (C), and for DGCR8 and AGO2 (D) in protein extracts of db/db mice and non-diabetic (db/+) controls. Vinculin was used as loading control. (E) Immunofluorescence for DICER1 in the skin of db/db and db/+ mice (bars: 100 μ m). (F) Histogram of the intensity of DICER1 immunofluorescence staining in db/db and db/+ skin specimens (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Western blot on skin extracts confirmed a reduction of DGCR8, XPO5, DICER1 and AGO2 in diabetic mice as compared to non-diabetic controls (Fig. 2 C and D). DROSHA antibodies did not detect any specific signal in skin samples of both diabetic and non-diabetic mice in our experimental conditions. DICER1 expression was further analyzed by immunofluorescence on skin sections of db/db and db/+ mice and indicated a general reduction in staining intensity in the skin of diabetic mice (Fig. 2 E and F).

3.3. Pri-miRNA expression levels in diabetic and non-diabetic skin reflect differences found in miRNA expression

Having found a significant decrease in the expression levels of genes responsible for miRNA biogenesis in diabetic skin samples,

we hypothesized that non-processed miRNA precursors accumulate in diabetic skin cells as compared to non-diabetic ones. Quantification of pri-miRNAs for the same miRNAs analyzed for miRnome validation was carried out by real-time RT-PCR (Fig. 3 A and B). Contrary to our hypothesis, differences in pri-miRNA expression levels quite closely correlated with those found in mature miRNAs, with the only exception of pri-miR148 that was expressed at similar levels in db/db mice and db/+ controls.

3.4. Hypoxia and oxidative stress conditions down-modulate miRNA biogenesis genes in dermal fibroblasts

Diabetic skin microenvironment is subjected to reduced oxygen supply due to micro- and macro-angiopathy, and hypoxia plays an

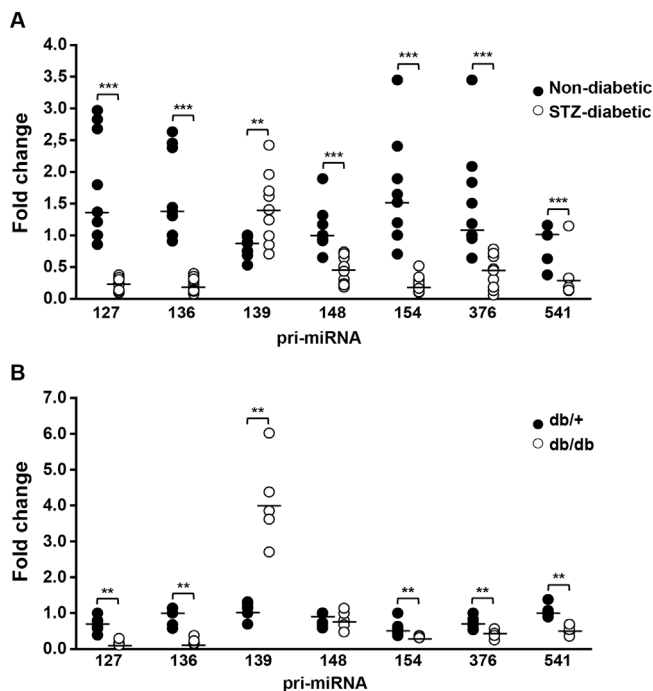


Fig. 3. Pri-miRNAs are also generally downregulated in the skin of diabetic mice. Real-time RT-PCR for pri-miRNAs tested for miRNome validation in skin RNA samples from streptozotocin-induced (STZ) diabetic mice and non-diabetic controls (A), or in db/db and non-diabetic controls (db/+) (B). TBP was used as reporter gene (** $p < 0.01$; *** $p < 0.001$).

important role in the development of diabetic complications [23]. The skin of animal models used in this study was shown to have reduced vascularization due to impaired angiogenic growth factor expression and altered endothelium responses [24,25].

A role for hypoxia in down-modulating DICER1 and DROSHA expression was reported in tumor contexts [26,27]. Therefore, we investigated whether the hypoxic condition could down-regulate the expression of miRNA-biogenesis genes also in the skin context. To this end, fibroblasts isolated from the skin of healthy donors were cultured in low oxygen levels (1% O_2) for 24 and 48 h. To verify whether culture conditions were able to induce a response to hypoxia, the expression of the transcription factor HIF-1 α was analyzed. In cells exposed to low O_2 for 48 h, HIF-1 α protein levels were strongly increased in all fibroblast strains (Fig. 4A).

MRNA levels of DGCR8, DROSHA, and XPO5 were significantly reduced after 24 h of culture in hypoxic condition (Fig. 4B). Proteins levels for the same genes were also found to be significantly reduced in extracts of fibroblasts cultured for 48 h under hypoxia, as compared to those maintained in normoxic conditions (Fig. 4C, D).

To test the effect of hyperglycemia on the expression of miRNA biogenesis genes, fibroblasts were cultured in 25 mM glucose for three weeks, a condition mimicking chronic hyperglycemia [28]. Fibroblasts cultured in high glucose did not display modulation of any of the genes analyzed, both at mRNA (Fig. 4E) and protein level (data not shown), as compared to those cultured in normoglycemic conditions.

As diabetes induces oxidative stress in the skin, the effect of oxidative stress on the expression levels of miRNA biogenesis genes was investigated by culturing fibroblasts in the presence of H_2O_2 . At first, cellular proliferation was evaluated by treating cells with increasing H_2O_2 amounts for 48 h (Fig. 5A). The minimal dose capable of significantly impairing cell growth, a feature of oxidative-stress induced cell senescence, was 200 μM H_2O_2 . The increase of total glutathione, a major cellular antioxidant, was

detected after 4 h of incubation with 200 μM H_2O_2 confirming the alteration of the redox status of cells (Fig. 5B). Therefore, this concentration was used to test the effect of oxidative stress on biogenesis genes. Real time RT-PCR performed in cells cultured for 24 h in the presence of H_2O_2 revealed a significant reduction in mRNAs only for DGCR8 (Fig. 5C). Decreased expression was confirmed for DGCR8 and observed for XPO5 at protein level after 48 h of H_2O_2 treatment (Fig. 5D, E).

4. Discussion

Hyperglycemic exposure leads to profound gene expression alterations that persist after reversal to a normoglycemic condition [29]. Such a hyperglycemic memory plays a central role in the recurrence of diabetic complications and it depends on epigenetic changes related to activity of advanced glycation end products (AGE), enhanced reactive oxygen species (ROS) and inflammatory mediators [30]. MiRNAs contribute to the control of tissue homeostasis by mediating post-transcriptional regulation of gene expression, and they were found to be involved in the development of diabetic skin complications [7].

Herein, we investigated miRNA expression in the skin by using experimental mouse models of type 1 and type 2 diabetes. The skin of these animals shows features which resemble the alteration observed in human diabetic skin with regard to epidermal differentiation, dermal composition, increased inflammation, reduced vascularization, neuropathy and delayed wound healing [24,25,31–34].

In the streptozotocin-induced diabetic mice, we found differences in miRNA expression between diabetic and non-diabetic skin. Differences were validated in a subset of miRNAs in the same strain of mice and in the type 2 diabetic mouse model. Among these miRNAs, some have proved target genes involved in pathways with important roles in regulating skin homeostasis and in promoting diabetic skin complications, such as Wnt, TGF- β , HIF-1 α , and VEGF-A signaling. HIF-1 α and VEGF-A are central players in the angiogenic response that is impaired in diabetic skin and that is a major cause of diabetic ulcer formation [35]. In the diabetic environment, HIF-1 α stabilization following hypoxia is impaired due to hyperglycemia [28,36], with reduction in expression levels for HIF-1 target genes. Among those genes there are VEGF-A and the chemokine stroma cell derived factor-1 (SDF-1), major players in the angiogenic response via endothelial cell activation and recruitment of endothelial progenitor cells [37]. On the other hand, in diabetic patients with ischemic cardiomyopathy, increased oxidative stress and signaling alterations induce a pseudo-hypoxia condition with mild chronic HIF-1 α activation [38]. Nevertheless, this constitutively active HIF-1 α is no longer sensitive to acute hypoxia.

Wnt and TGF- β pathways are also involved in skin wound healing and play a role in tissue remodeling and extracellular matrix deposition [39–41], other aspects altered in diabetic skin. Moreover, Wnt is directly involved in insulin homeostasis, with opposing roles ascribed to canonical and non-canonical Wnt signaling [42].

Different gene pathways found to be regulated by differentially expressed miRNAs are involved in neuronal function; this is not surprising, as peripheral neuropathy is a major defect of diabetic skin, with important consequences for foot ulcer formation [43].

Interestingly, a global miRNA down-modulation was observed in diabetic compared to non-diabetic skin, suggesting that the diabetic skin microenvironment affects miRNA biogenesis. miRNA biogenesis is a multistep process involving several molecular mediators. Most miRNAs are processed via the canonical biogenesis pathway. Non-canonical pathways involve the same proteins of the canonical one, in combination with other proteins,

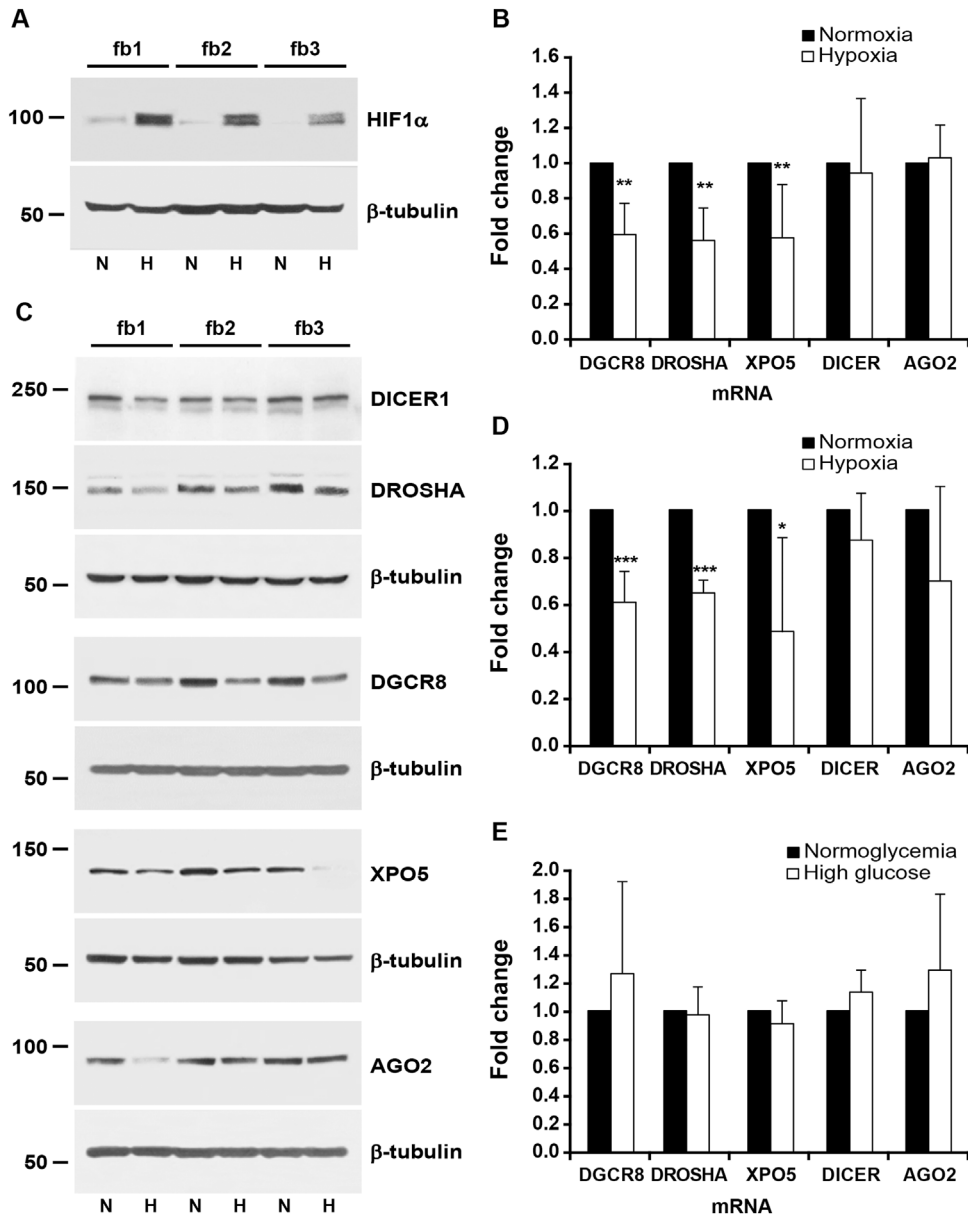


Fig. 4. Hypoxia down-modulates the expression levels of miRNA biogenesis genes in dermal fibroblasts. (A) Western blot for HIF-1 α to check hypoxic conditions in protein extracts of primary normal human dermal fibroblasts cultured for 48 h in conditions mimicking hypoxia. β -tubulin was analyzed as loading control. (B) Real-time RT-PCR for miRNA biogenesis genes in primary human normal dermal fibroblasts cultured for 24 h in 1% O₂. TBP was used as reporter gene. (C) Protein levels of miRNA biogenesis genes in human dermal fibroblasts following 48 h culture in the presence of 1% O₂. Expression of β -tubulin was used as loading control. (D) Densitometry of western blot data on miRNA biogenesis gene expression. (E) Real-time RT-PCR for biogenesis genes in human primary dermal fibroblasts cultured in the presence of high glucose levels. TBP was used as reporter gene. N = normoxia; H = hypoxia (* p < 0.05; ** p < 0.01; *** p < 0.001).

but not all biogenesis proteins are involved in a single non-canonical pathway. We analyzed a subset of genes involved in the different steps of the canonical pathway: DROSHA, DGCR8, XPO5, DICER1 and AGO2. All of them were expressed at significantly reduced levels in the skin of both streptozotocin-dependent and db/db diabetic mice, suggesting that reduced biogenesis gene levels account for altered miRNA expression in diabetic skin. Despite the observed reduced expression of biogenesis proteins, a minority of miRNAs were up-regulated in diabetic skin. Processing by non-canonical biogenesis pathways cannot explain the increased expression of these miRNAs, as all biogenesis genes analyzed were found to be down-regulated in diabetic skin. A hypothetic explanation is that the higher levels of a minority of miRNAs can be due to a transcriptional increase that overcomes the decrease in the efficiency of miRNA precursor processing.

MiRNAs found to be significantly down-regulated do not correspond to those that were reported to play a role in diabetic complications [8]. Our analysis, therefore, highlights novel miRNAs with potential role in skin homeostasis to be further investigated.

In cancer cells, deregulated miRNA biogenesis was correlated with hypoxic condition within the tumor and with pri-miR accumulation in cell nuclei [26,27]. Dermal human fibroblasts cultured in hypoxic conditions expressed reduced levels of DGCR8, DROSHA and XPO5, indicating that hypoxia down-regulates miRNA biogenesis genes in skin cells. The hypoxic conditions used in our experimental setting resemble severe acute ischemia found in chronic wounds. This data suggests that miRNA biogenesis gene down-modulation may have implications in the hypoxic environment of diabetic foot ulcers. Dermal fibroblasts cultured in the presence of H₂O₂ express reduced levels of DGCR8

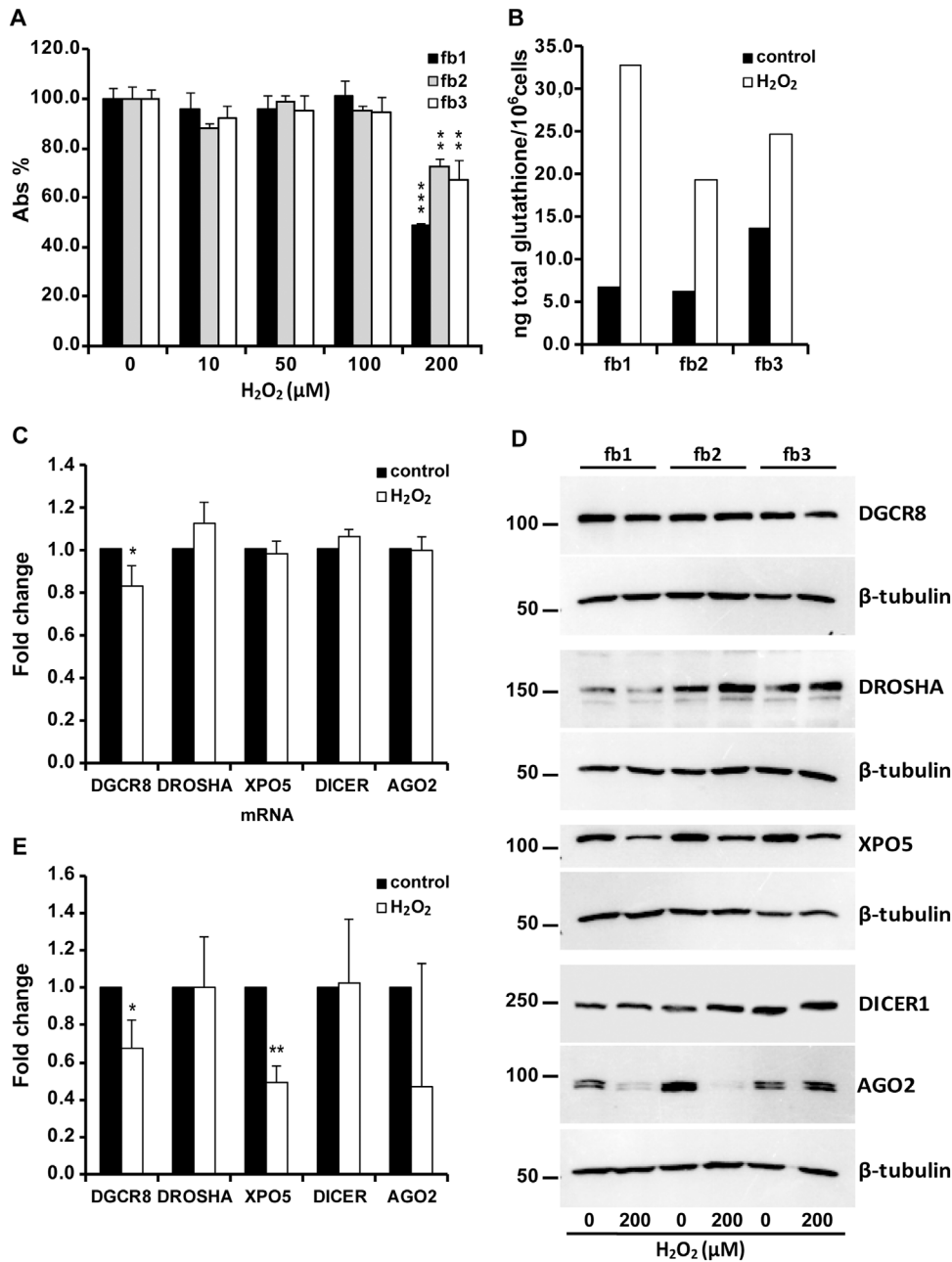


Fig. 5. Effects of oxidative stress on expression levels of miRNA biogenesis genes in dermal fibroblasts. (A) Dose-dependent effect of H_2O_2 on fibroblast proliferation after 48 h of treatment. (B) Total glutathione amounts relative to the cell numbers in fibroblasts treated with 200 μM H_2O_2 for 4 h. (C) Real-time RT-PCR for miRNA biogenesis genes in primary human normal dermal fibroblasts cultured for 24 h in 200 μM H_2O_2 . TBP was used as reporter gene. (D) Western blot results of fibroblasts cultured for 48 h in conditions mimicking the oxidative stress (i.e. 200 μM H_2O_2). β -tubulin was used as loading control. (E) Densitometric analysis of western blot data on miRNA biogenesis proteins (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

and XPO5, indicating that increased ROS activity may contribute to the observed reduction in genes responsible for miRNA biogenesis in this cell type. We can not exclude that alterations observed in miRNA biogenesis genes and modulation of their expression under hypoxic and oxidative stress conditions may also occur in other skin cell types.

The expression of pri-miRNAs for the same miRNAs analyzed for miRnome validation revealed that differences in expression levels between diabetic and non-diabetic samples are similar to those found for mature miRNAs. This result suggests that reduced miRNA levels are due to a more general transcriptional impairment that also affects pri-miRNA transcription.

In conclusion, our data confirm the complexity of molecular defects in diabetes, and that central mechanisms involved in the regulation of gene transcription are perturbed. They reinforce the concept that acting on the molecular machinery regulating gene expression can be pursued as a therapeutic option for diabetic complications.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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