Squamous cell carcinoma of the skin shows a distinct microRNA profile modulated by UV radiation

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Running title: microRNAs in skin SCC

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Cutaneous squamous cell carcinoma (SCC) is the second most common skin malignancy in the general population. There are many risk factors for SCC, the most important one being solar radiation. The incidence of SCC is increased 60-100 fold in organ transplant recipients (OTR), making it the most common malignancy in these patients (Berg and Otley, 2002; Euvrard *et al.*, 2003). SCC in OTR is characterized by a higher risk of metastasis in up to 20% and shows a more aggressive course than SCC in the general population (Euvrard *et al.*, 2003).

Recent work has delineated a class of small non-coding RNA species known as microRNAs. By associating with the 3'-UTR region of target mRNAs, microRNAs have important regulatory roles in diverse cellular pathways including development, differentiation, organogenesis, stem cell and germ line proliferation and apoptosis (Chen *et al.*, 2004; Lin, 2005; O'Donnell *et al.*, 2005; Poy *et al.*, 2004; Zhao *et al.*, 2005). Importantly, the successful use of antagomirs to silence microRNAs in mice and nonhuman primates suggests a possible therapeutic use of microRNAs (Elmen *et al.*, 2008; Krutzfeldt *et al.*, 2005).

Although SCC is one of the most common cancers, no studies so far have focused on microRNA expression and function in this particular tumor. We looked at the expression of candidate microRNAs with at least partially described functions in keratinocytes: miR-21, miR-184 for both of which oncogenic properties have been reported, miR-203 as keratinocyte-specific microRNA, and miR-205 as antagonist of miR-184, in the immunocompetent population and in OTR. As UV radiation is the most important risk factor for SCC, we also investigated the influence of UV radiation on the expression of these miRs in normal human keratinocytes.

Analyses were performed on SCC tissue material from 13 OTRs, 17 immunocompetent patients and 7 normal skin samples obtained from healthy individuals. Treatment of OTR consisted of Cyclosporine A in combination with other immunosuppressive drugs. All SCC samples were diagnosed as well or moderately differentiated SCCs by a board-certified dermatohistopathologist (Supplemental Table 1). The post-hoc power analysis for statistically significant differences reached 60-98%, respectively.

Real-time RT-PCR analysis showed significantly increased expression of miR-21. This finding extends the list of tumors with upregulated miR-21, and suggests that miR-21 may play an essential role in development or maintenance of SCC of the skin. Similarly SCC showed increased expression of miR-184. Interestingly, miR-184 was barely detectable in normal epidermis, but much increased in SCC.

Similar to our findings for miR-184 expression in epidermis, we could not detect this microRNA in normal human keratinocytes. Interestingly, miR-184 was recently shown to be upregulated in tongue SCC with strong data supporting the hypothesis that this microRNA may induce cell transformation and carcinogenesis (Wong *et al.*, 2008). In our samples miR-205 did not seem to be important for SCC judging by its expression, while its function may be important and may well be modulated my miR-184 which we found to be elevated in SCC. Expression of miR-205 may thus be considered as a control for other measurements.

Although miR-203 was described in the context of cell senescence which could suggest an increased expression in the older population, we found its expression decreased in the SCC group (i.e. the older group), suggesting differences to represent tumor-associated features such as an increased stem cell population and higher proliferative capacity rather than mere aging. It is known that SCC contains a higher percentage of highly proliferative, undifferentiated cells than normal skin. miR-203 antagonizes expression of p63 which is a transcription factor playing an essential role in the maintenance of "stemness" in the skin and which is known to be upregulated in SCC (Dotto and Glusac, 2006; Lena *et al.*, 2008). Our data suggest that decreased miR-203 may unleash p63 expression, leading to decreased cell senescence and supporting SCC formation.

Expression levels of these selected microRNAs were similar between well and moderately differentiated SCC (Fig. 1b).

As long-term drug-induced immunosuppression is closely linked to greatly increased SCC formation (Caforio *et al.*, 2000), we compared the expression of the four microRNAs under investigation in SCC derived from immunocompetent patients and OTR (Fig. 1c) but did not find any significant differences. *In vitro* analysis of miR-21, miR-203, miR-205 and miR-184 expression in primary human keratinocytes exposed to different concentrations of CsA supported these findings (Suppl. Fig. 1): We did not observe any influence of CsA on these microRNA expression levels. The greatly increased incidence of SCC in OTR seems thus unlikely to be mediated by these miRs directly. It is tempting to speculate that any SCC-promoting effects of miRs will have a greater impact in the context of a reduced tumor defense in OTR, thus potentially indirectly contributing to the greatly increased cutaneous carcinogenesis in OTR.

We found that UV irradiation alters miR expression. We observed that UVA radiation increased expression of miR-21, miR-203 and miR-205 (Fig. 2a). UVB radiation, however, increased expression of miR-203, slightly but significantly decreased expression of miR-205 and had no effect on miR-21. Interestingly, UVA, but not UVB increased the expression of miR-21, until now the best described microRNA with potent carcinogenic properties. This finding is particularly interesting as UVA radiation is responsible for SCC formation to a much higher extent than UVB. The mechanism by which UVA radiation upregulates miR-21 expression may further explain the differential influence of these two wavelengths on SCC formation. Both UVA and UVB induce miR-203 expression, possibly in line with differentiation and aging of keratinocytes after solar irradiation. The diverging effects of UVA and UVB radiation on miR-205 expression, however, underline different mechanisms of cell damage mediated by these wavelengths. The consequence of miR-205 induction or down-modulation in skin keratinocytes remains to be investigated.

Topical non-steroidal anti-inflammatory drugs such as diclofenac are effective against early SCC and are the subject of attempts to prevent UV-mediated SCC formation (Ortonne *et al.*, 2006). In our study however, diclofenac does not impact microRNA expression (Suppl. Fig. 2). This speaks against the cyclo-oxygenase pathway as a major modulator for selected miR expression induced by UV.

In summary, we show that expression of miR-21, miR-184 and miR-205 is changed in SCC compared to normal skin. UV, in particular UVA as major risk factor for SCC, impacts miR expression, suggesting an early role for these miRs in SCC formation, possibly affecting such important processes as differentiation and apoptosis while the calcineurin and cyclo-oxygenase pathways seem uninvolved.

Conflict of Interest

The authors state no conflict of interest.

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Figure legends

Figure 1

Altered microRNA expression in skin SCC. (a) RealTime PCR showed increased expression of miR-21 and miR-184 and decreased expression of miR-203 in cutaneous SCC as compared to normal skin. There was no detectable difference in expression of miR-205. Further analysis of SCC group according to histological differentiation (b) and drug-induced immunosuppression in OTR (c) did not reveal any significant changes in the expression of these selected microRNAs. (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

Figure 2

UVA and UVB irradiation differentially influence miRs expression in normal keratinocytes. (a) UVA irradiation significantly increases expression of miR-21, miR-203 and miR-205, while (b) UVB has no effect on miR-21, upregulates miR-203 and downregulates miR-205 expression. miR-184 was not detected in normal keratinocytes before and after irradiation.

Figure1

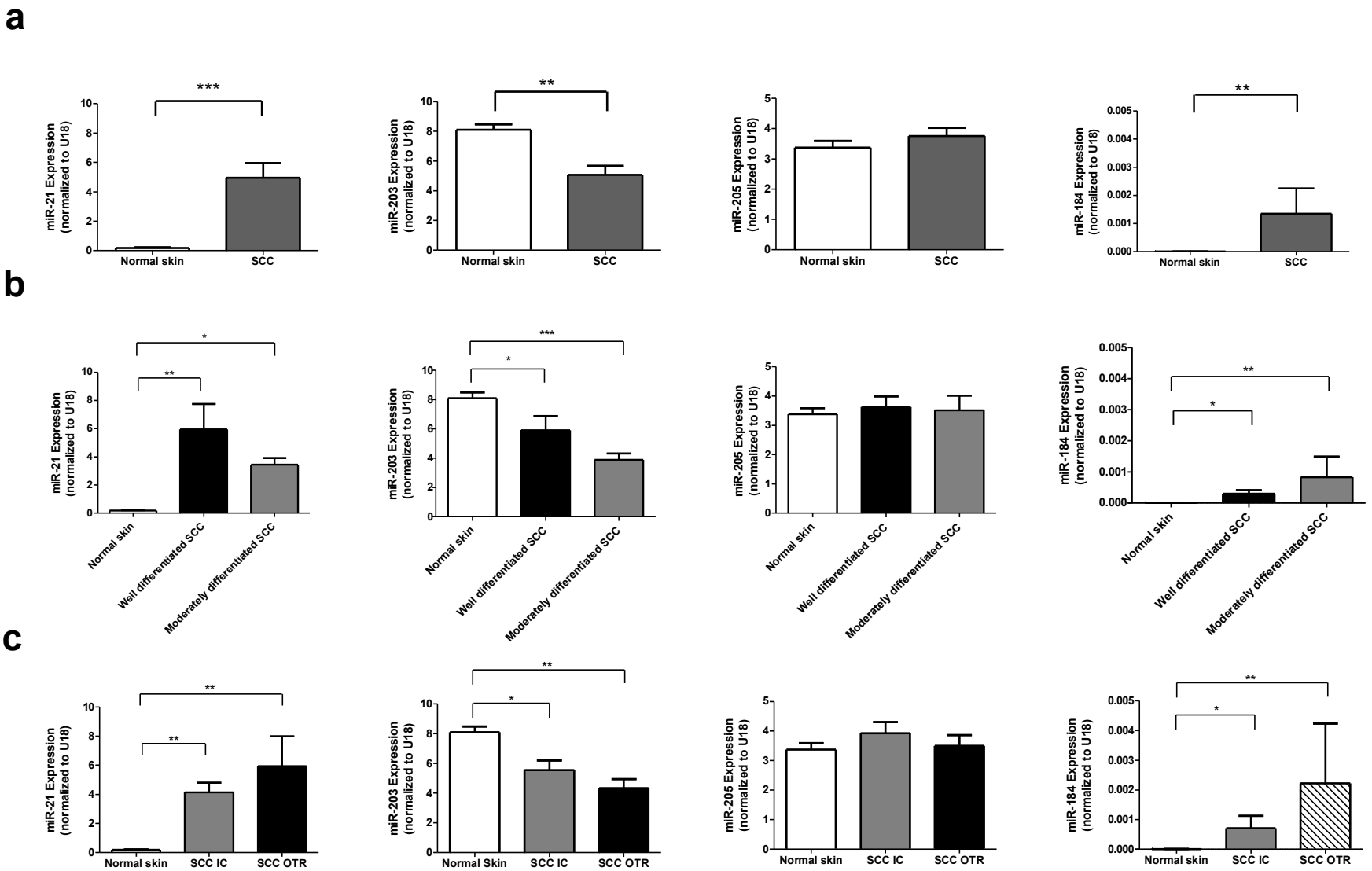
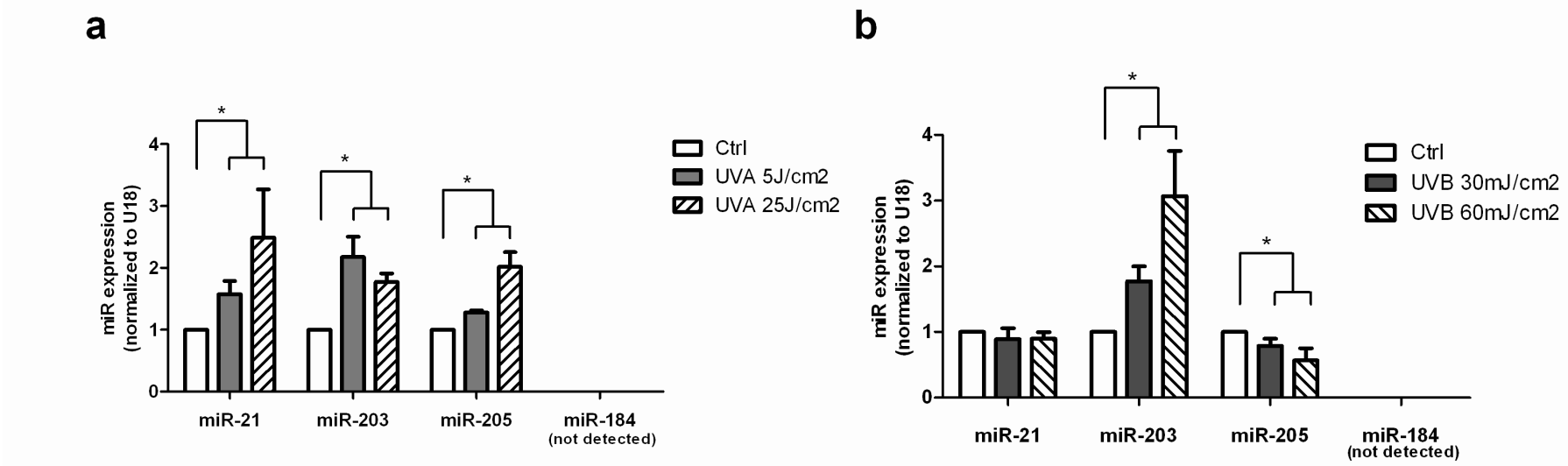

a
b
c
miR-21 Expression (normalized to U18)
miR-203 Expression (normalized to U18)
miR-205 Expression (normalized to U18)
miR-184 Expression (normalized to U18)
Normal skin
SCC
Well differentiated SCC
Moderately differentiated SCC
Normal Skin
SCC IC
SCC OTR

Figure 2

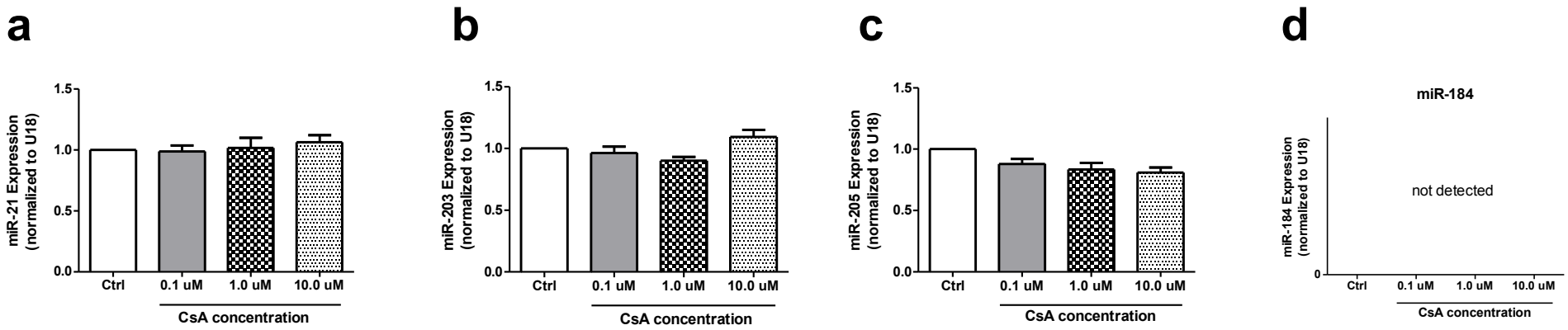

a
b
miR expression
(normalized to U18)
Ctrl
UVA 5J/cm2
UVA 25J/cm2
UVB 30mJ/cm2
UVB 60mJ/cm2
miR-21
miR-203
miR-205
miR-184
(not detected)
*
0
1
2
3
4

Baseline Characteristics of Subjects		IC	OTR	Normal skin	Total
Patients		17	13	7	37
Gender	Male [n (%)]	11 (65)	12 (92)	1 (14)	24 (69)
Age (y)	Age [mean (± SD)]	80.29 (±16.59)	66.54 (±7.96)	46.6 (±10.36)	70.37 (±17.39)
	Range	52-101	50-80	35-58	35-101
Organ transplant	Kidney		8		
	Heart		4		
	Lung		1		

Supplemental Table 1

Baseline characteristics of patients; n – number, y – years, IC – immunocompetent, OTR – organ transplant recipients, SD – standard deviation.

Supplemental Figure 1

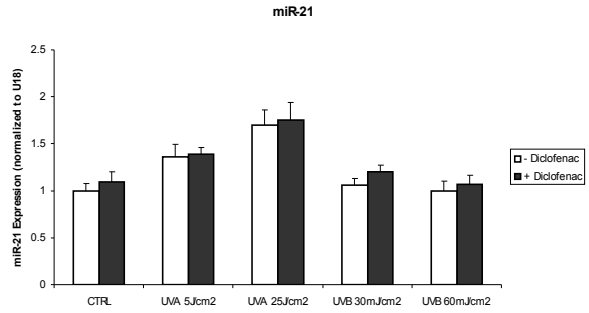


Supplemental Figure 1

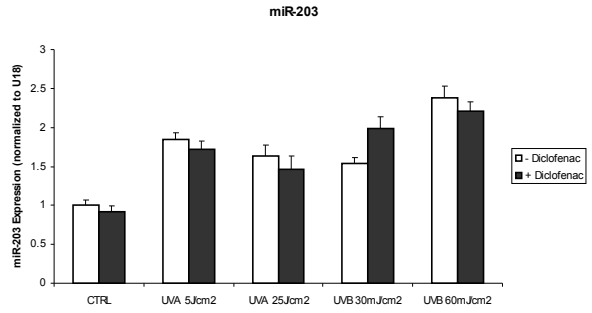
Cyclosporine A treatment does not affect the expression of (a) miR-21, (b) miR-203 and (c) miR-205. Normal human keratinocytes were incubated with different concentrations of CsA (0.1 uM, 1.0 uM and 10.0 uM) for 12 hours. Total RNA was then isolated, and real-time RT-PCR was performed. miR-184 was not detected in normal human keratinocytes with and without CsA treatment (d).

Supplemental Figure 2

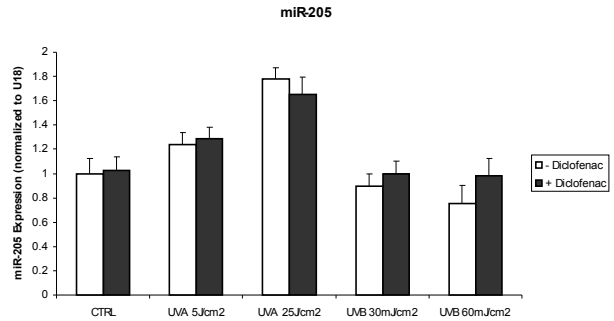
a



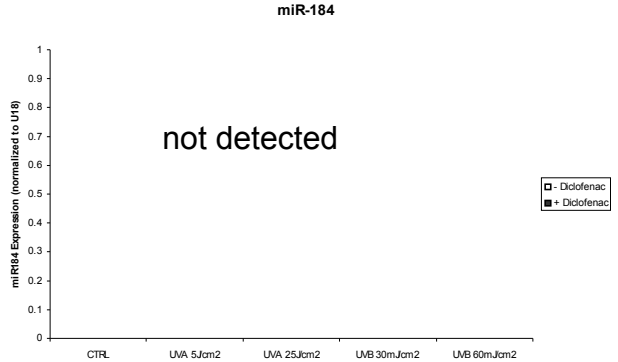
b



c



d



Supplemental Figure 2

Diclofenac does not change the expression of (a) miR-21, (b) miR-203 and (c) miR205, and does not influence effect of UVA and UVB irradiation on expression of these miRs in normal human keratinocytes. (d) miR-184 was not detected in both irradiated and control keratinocytes with and without diclofenac treatment. For this experiment cells were incubated for 2h with 10uM Diclofenac (a concentration that was shown to have inhibitory effects on cyclooxygenase activity (Mitchell *et al.*, 1993)) and then exposed to UVA or UVB in two different doses (5 J/cm2 and 25 J/cm2 or 30 mJ/cm2 and 60 mJ/cm2 respectively). Cells were then further incubated with the same concentration of diclofenac for 6 hours, when total RNA was extracted for real-time RT-PCR.