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## **Antioxidant properties of small proline-rich proteins : from epidermal cornification to global ROS detoxification and wound healing**

Vermeij, W.P.

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# **Antioxidant properties of small proline-rich proteins**

*from epidermal cornification to global ROS  
detoxification and wound healing*

**Wilbert Vermeij**

The research presented in this thesis was performed at the department of Molecular Genetics, Leiden Institute of Chemistry, Leiden University, The Netherlands.

Cover: The Matusевич Glacier flowing towards the eastern coast of Antarctica. Image was taken by the Earth Observatory, EO-1 Advanced Land Imager, NASA.

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# **Antioxidant properties of small proline-rich proteins**

*from epidermal cornification to global ROS  
detoxification and wound healing*

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## Thesis Outline

In 1988, two highly homologous groups of genes were identified in human epidermal keratinocytes. Their expression levels were increased both in response to UV-light and during epidermal differentiation. The encoded proteins were small, contained repeated sequences and were exceptionally rich in proline residues and were therefore designated as small proline-rich (SPRR) proteins. Today, 11 members of this gene family are known. They are categorised into four groups, based on sequence homology and the amount of internal repeats. During the establishment of the epidermal barrier SPRR proteins are expressed in the upper layers of the epidermis. Their well-known function in the skin's barrier formation and adaptation is reviewed in *Chapter I*.

The various SPRR family members are differentially expressed in squamous epithelia and respond differently to external stressors. Their promoter regions contain a dedicated mixture of transcription factor binding sites that allows this divergent gene expression. In *Chapter II* the cooperative gene regulation by two of these transcription factors, namely Skn-1a and Ese-1, is presented.

A few years ago, SPRR proteins were unexpectedly found in all major tissues, ranging from gut and brain to liver and heart. While analysing wounded skin we found an important role for the SPRR proteins in the healing process. Directly after wounding, reactive oxygen species (ROS) are generated as the initial signal that activates the immune response and as a defence against invading bacteria. However, ROS are also harmful for the surrounding tissue and impede subsequent wound closure. SPRR proteins can directly reduce the toxic ROS levels in the adjacent cells and thereby promote cell migration. This study is described in *Chapter III*. Apparently, this novel role in wound healing is far more widespread than their established function in skin cornification.

In *Chapter IV*, the antioxidant properties of the SPRR proteins were extended to non-wounded skin. As the amount of oxygen in air is almost 7 times higher than within our body a specialised barrier is required to protect us from oxidation. We showed that the SPRR proteins, during their conventional role in the formation of a mechanical and permeability barrier, also provide an antioxidant barrier to our skin. Thus, the SPRR proteins directly function as our first line of defence against ROS.

In *Chapter V*, a screen for SPRR protein interactions partners is presented. The role of some identified proteins confirmed a role of SPRRs in cornification and antioxidant function, but also revealed a role in DNA-binding, which was confirmed by direct experimentation. Furthermore, a molecular model explaining how the intracellular oxidation state of SPRRs likely influences their selective protective function is provided.



# Chapter I

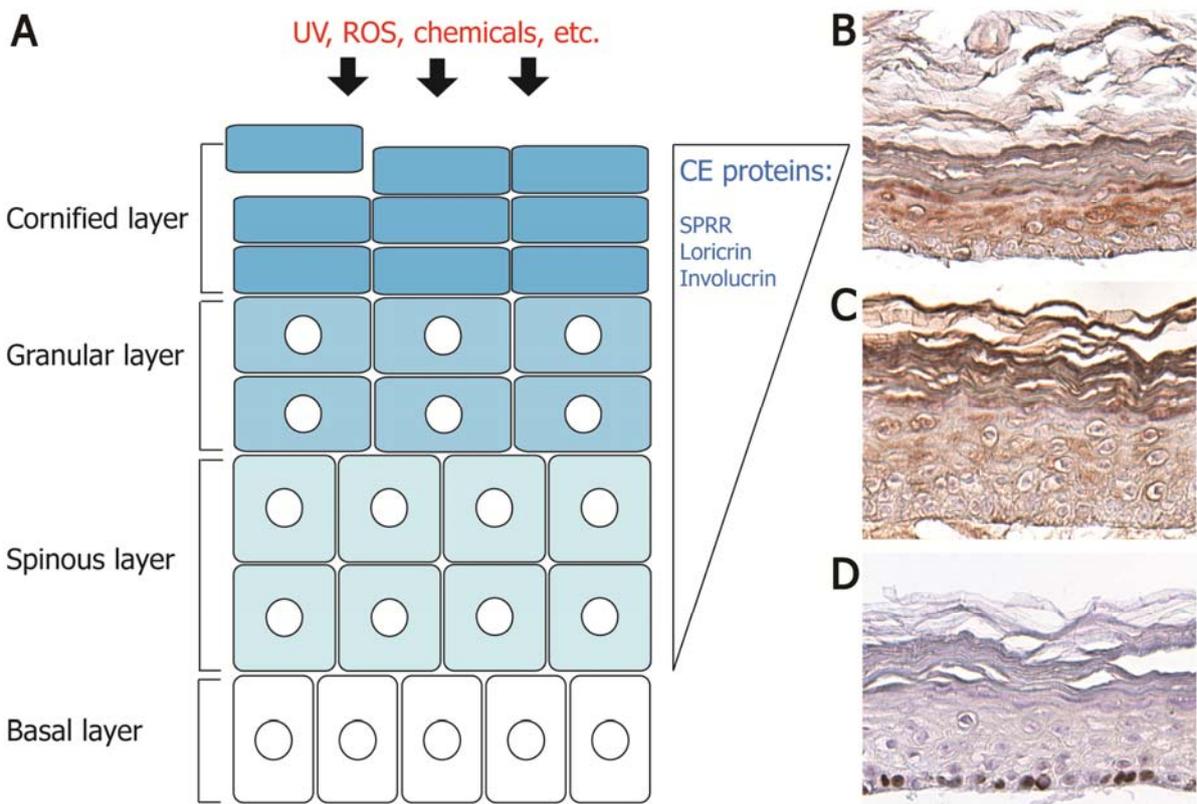
*SPRR proteins: from epidermal cornification to global ROS detoxification and wound healing*

*General Introduction*

## SPRR proteins: from epidermal cornification to global ROS detoxification and wound healing

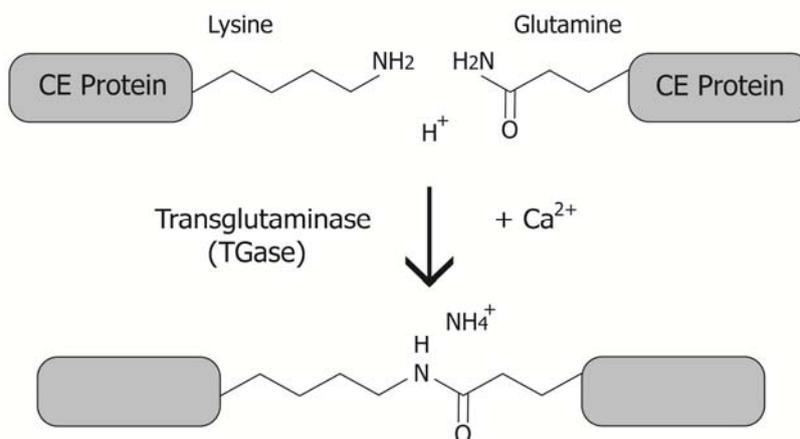
### Protective skin barrier

At the skin surface, the epidermis functions as primary barrier against a continuous challenge of various environmental hazards and is essential for mammalian life<sup>19,84</sup>. It excludes harmful microorganisms, withstands mechanical and chemical assaults, and protects our body from dehydration<sup>19,57</sup>. It is a thin multi-layered compartment comprised of a basal-, spinous-, granular-, and cornified layer, respectively (Figure 1). The major barrier property resides within the cornified layer<sup>44</sup>, a layer of dead flattened cells on the skin surface. These cells are in direct contact with atmospheric oxygen and constitute a first line of defence<sup>163</sup>. They contain a special structure beneath the plasma membrane, termed the cornified cell envelope (CE), which is comprised of cross-linked proteins and lipids<sup>68,130,147</sup>. Although these cells will eventually shed off, the skin's barrier is constantly self-renewed<sup>39,98</sup>.



**Figure 1:** Protective epidermal barrier. A, Graphical representation of a cross section of the multi-layered human skin. The main barrier resides within the outermost cornified layer. These cells contain an insoluble protein structure of cross-linked cornified envelope precursor proteins, such as involucrin, loricrin and the SPRR protein family members. B-D, Cultured human skin equivalents stained by immunohistochemistry for SPRR1 (B), SPRR2 (C), and Ki67 (D). SPRR protein expression (brown-staining) is observed in the upper epidermal layers while Ki67 (a marker for dividing cells) is restricted to some cells of the basal layer.

Within the innermost basal layer a population of mitotically active keratinocytes (Figure 1D) provides a continuous supply of new cells under both homeostatic and injury conditions<sup>57,198</sup>. Keratinocytes from this basal layer divide asymmetrically to generate two daughter cells of which one retains a proliferative basal cell character while the second becomes a committed suprabasal cell<sup>101</sup>. This cell undergoes a process called cornification and starts to migrate upwards through the different skin layers in a period of 4 to 5 weeks. Throughout this process the cell undergoes several morphological changes and at each stage of the cornification process a different subset of genes is expressed. During stage one (initiation), envoplakin<sup>154</sup>, periplakin<sup>153</sup> and involucrin<sup>148</sup>, are expressed within the spinous layer. They are directed to the plasma membrane and initiate the assembly of the CE<sup>83,176</sup>. Subsequently, these proteins are cross-linked by transglutaminases (Figure 2)<sup>41,111</sup>. In the granular layer they are covalently attached to the desmosomal junctions to form a scaffold for other cornified envelope precursor proteins<sup>84,175</sup>. Concomitantly a complex series of lipids are synthesised (stage two; lipid-envelope formation). These lipids are coated around the protein envelope scaffold and ultimately form a 5 nm thick lipid envelope<sup>19,130</sup>. At stage three (reinforcement) more cornified envelope precursor proteins (e.g. loricrin<sup>123</sup>, filaggrin<sup>149</sup>, repetin<sup>100</sup>, trichohyalin<sup>104</sup> and the small proline-rich (SPRR)<sup>18,60,86</sup>, late cornified envelope (LCE)<sup>77,116</sup> or S100<sup>45</sup> protein families) are cross-linked to the pre-existing scaffold. This protein envelope (of approximately 10 nm thick) together with the lipid envelope turns the CE into an extremely tough structure which still allows the high flexibility of our skin.



**Figure 2:** Schematic overview of the protein cross-linking reaction by transglutaminases. Two CE proteins are cross-linked together via a lysine residue on protein 1 and a glutamine residue on protein 2 by the calcium-dependent transglutaminase enzyme.

### Epidermal Differentiation Complex

Most of the cornified envelope precursor genes are located on a small region on human chromosome 1q21, also known as the epidermal differentiation complex (EDC)<sup>126</sup>. The genes in this region are co-ordinately regulated during the cornification process. For example, the single cornification genes involucrin and loricrin as well as the SPRR and LCE clusters map to a 2.5 Mbp region<sup>84,126,193</sup>. These proteins all share similar head and tail

domains which are, due to their high lysine and glutamine content, used for transglutaminase cross-linking<sup>10</sup>. The internal domains do not show any sequence homology and are unique for each (specific type of) protein(s). Due to their common location on the EDC, their involvement in the cornification process and the high sequence homology of their external domains, it is generally believed that all genes in this region originated from duplications of a single ancestor gene which later has diverged<sup>10,190</sup>.

Furthermore, the middle part of all above mentioned EDC genes contains typical repetitive sequences. The central domain of involucrin consists of 39 repeats of six different amino acids and the whole protein appears to originate from a single CAG repeat<sup>40</sup>. These repeats contain mostly charged residues, which makes involucrin a highly soluble protein<sup>40,189</sup>. The middle part of loricrin has one of the highest known contents of glycine residues which are configured in quasipeptide repeats. As a result, loricrin is an extremely flexible but also poorly soluble protein<sup>84,123</sup>. The SPRR family members all have proline-rich central domains (Figure 3). This family consists of 11 highly homologous members which are subdivided into four groups (Table 1). Each group contains a different amount of tandem repeats, varying by 8 or 9 amino acids in size. With an overall proline content of approximately 30%, the SPRR proteins have a very rigid protein backbone<sup>18,60</sup>. The group of LCE genes appear to be hybrids between loricrin and SPRR<sup>84</sup>.

**Table 1:** Statistics of human SPRR proteins

Protein	Amino acids	Size (kDa)	# of Repeats	% Proline	% Lysine	% Glutamine	% Cysteine
SPRR1A	89	9,88	6	30,3	12,4	20,2	9
SPRR1B#	89	9,89	6	29,2	12,4	18	9
SPRR2A#	72	7,96	3	37,5	11,1	16,7	15,3
SPRR2B	72	7,96	3	38,9	11,1	16,7	15,3
SPRR2C*	72	7,94	3	34,7	11,1	15,3	15,3
SPRR2D	72	7,9	3	37,5	11,1	16,7	16,7
SPRR2E	72	7,85	3	38,9	11,1	16,7	18,1
SPRR2F	72	7,8	3	36,1	11,1	16,7	18,1
SPRR2G	73	8,1	3	39,7	9,6	13,7	15,1
SPRR3#	169	18,1	16	22,5	11,8	10,1	4,7
SPRR4#	79	8,7	4	16,5	13,9	29,1	8,9

#Protein sequences, repeats, predicted secondary structure and diverse specific amino acids of SPRR1B, SPRR2A, SPRR3, and SPRR4 are represented in more detail in Figure 3.

\*Truncated translation due to premature stop codon.

### Barrier adaptation by SPRR

The protein composition of the CE varies between different body sites. Although it always comprises a total of 85-90% loricrin and SPRR proteins, their relative molar ratio ranges from >100-1 in trunk epidermis to 5-1 in footpad epidermis and 3-1 in murine



(e.g. retinoic acid or TPA treatment)<sup>2,17,29,58,59,61,74,75,81,82,86,96,110,120,160,185,203,204</sup>. Promoter region analysis revealed the existence of a great diversity in regulatory elements for the different family members. They all contain a dedicated mixture of AP-1, Ets, ATF, ISRE, octamer, and/or zinc finger transcription factor binding sites<sup>16,18,50-52,136,159</sup>. This versatility in regulatory elements explains the observed divergent gene expression. In this way, the SPRR proteins can be rapidly produced due to existence of multiple genes and a high flexibility in the overall protein dosage is allowed<sup>18</sup>.

### Global SPRR expression

Ten years ago, SPRR expression was unexpectedly detected in non-squamous epithelia. Hooper and co-workers analysed the host response of the intestine to microorganism colonisation at a transcriptional level<sup>73</sup>. By using DNA microarrays they compared the gene expression profile of the intestine of germ-free mice with intestines colonised by various members of the microflora. The most pronounced response was the increase in SPRR2A by more than 200 fold while all other observed genes were only affected up to 10 fold<sup>73</sup>. This response, however, was specific for certain members of the microflora. The identified genes revealed that the combination of microorganisms in our microflora can affect important intestinal functions such as metabolism, nutrient absorption and angiogenesis<sup>73</sup>. At the same time, analysis of the adaptive response of the remaining intestinal tissue after small bowel resection revealed again the highest expressional change for SPRR2A<sup>177</sup>.

**Table 2:** Upregulation of SPRR proteins identified in non-squamous epithelia

Study	SPRR	Organ	Organism	Upregulated due to
Ding <sup>36</sup>	SPRR1A	Circulatory system	mice	Dilated cardiomyopathy
Pradervand <sup>140</sup>	SPRR1A,2A,2B	Circulatory system	mice	Ischemic stress
Pyle <sup>143</sup>	SPRR3	Circulatory system	human & mice	Cyclic mechanical strain
Pyle <sup>144</sup>	SPRR3	Circulatory system	human	Cyclic biomechanical stress
Young <sup>206</sup>	SPRR1A,1B,2J,3	Circulatory system	human & mice	Atherosclerotic plaques
Abgueguen <sup>1</sup>	SPRR2A	Digestive system	mice	Iron overload
Bracken <sup>14</sup>	SPRR3	Digestive system	human	Inflammatory disorder
Demetris <sup>32</sup>	SPRR2A	Digestive system	mice	Bile duct ligation
Demetris <sup>33</sup>	SPRR2A	Digestive system	mice	Biliary barrier defects
Demetris <sup>34</sup>	SPRR2A	Digestive system	mice	Bile duct ligation
Hooper <sup>73</sup>	SPRR2A	Digestive system	mice	Microflora colonisation
Knight <sup>92</sup>	SPRR2A	Digestive system	mice	Nematode infection
Mueller <sup>129</sup>	SPRR2A	Digestive system	mice	Helicobacter infection
Nozaki <sup>133</sup>	SPRR2A,2B,2E, 2I	Digestive system	human & mice	Bile duct ligation
Park <sup>135</sup>	SPRR2A	Digestive system	mice	Embryonic development
Ren <sup>146</sup>	SPRR2A	Digestive system	mice	Electromagnetic pulses
Stern <sup>177</sup>	SPRR2A	Digestive system	mice	Small bowel resection
Suda <sup>179</sup>	SPRR3	Digestive system	human	Vertical tooth movement

Study	SPRR	Organ	Organism	Upregulated due to
Sun <sup>181</sup>	SPRR2A	Digestive system	mice	Bacterial infection
Chen <sup>23</sup>	SPRR1B	Eye	human & mice	Sjögren syndrome
Chen <sup>24</sup>	SPRR1B,2A	Eye	human	Osmotic stress
De Paiva <sup>30</sup>	SPRR2	Eye	mice	Desiccating stress
De Paiva <sup>31</sup>	SPRR2	Eye	mice	Desiccating stress
Kawasaki <sup>88</sup>	SPRR2A	Eye	human	Sjögren syndrome
Li <sup>106</sup>	SPRR1B	Eye	human & mice	Dry eye disease
Tong <sup>188</sup>	SPRR1A,1B,3	Eye	human	Pterygium
Gotter <sup>63</sup>	SPRR1B	Lymphoid system	human	Thymic expression
Bonilla <sup>13</sup>	SPRR1A	Nervous system	mice	Peripheral axonal damage
Carmichael <sup>21</sup>	SPRR1	Nervous system	rat	Stroke
Fischer <sup>49</sup>	SPRR1	Nervous system	rat	Optic nerve injury
Li <sup>107</sup>	SPRR1A	Nervous system	mice	Spinal cord injury
Lobsiger <sup>108</sup>	SPRR1A	Nervous system	mice	Amyotrophic lateral sclerosis
Marklund <sup>114</sup>	SPRR1A	Nervous system	rat	Traumatic brain injury
Starkey <sup>171</sup>	SPRR1A	Nervous system	mice	Peripheral nerve injury
Hong <sup>71</sup>	SPRR2A,2H	Reproductive system	mice	Estrogen treatment
Hong <sup>72</sup>	SPRR2A,2B,2C,2D,2E,2F,2G	Reproductive system	mice	Estrogen treatment
Kouros-Mehr <sup>99</sup>	SPRR1A	Reproductive system	mice	Mammary branching morphogenesis
Mercier <sup>124</sup>	SPRR1A	Reproductive system	mice	Estrogen treatment
Moggs <sup>127</sup>	SPRR1A,2A,2C,2E,2F,2G,2I,2J	Reproductive system	mice	Estrogen treatment
Morris <sup>128</sup>	SPRR1A,2A,2B	Reproductive system	mice	Mammary gland development
Robertson <sup>150</sup>	SPRR2A	Reproductive system	mice	Prostate development
Tan <sup>182</sup>	SPRR2A,2I	Reproductive system	mice	Oestrous cycle
Tan <sup>183</sup>	SPRR2A,2B,2D,2E,2F,2G,2K	Reproductive system	mice	Oestrous cycle
Tesfaigzi <sup>184</sup>	SPRR1	Reproductive system	hamster	Cell division
Domachowske <sup>37</sup>	SPRR1A	Respiratory system	mice	Viral infection
Rouse <sup>152</sup>	SPRR2A	Respiratory system	mice	Tobacco smoke and Ovalbumin
Sandler <sup>157</sup>	SPRR	Respiratory system	mice	Parasite eggs
Vos <sup>195</sup>	SPRR1A,1B,2A	Respiratory system	human	Pro-inflammatory cytokines
Yoneda <sup>205</sup>	SPRR1B	Respiratory system	human	Smoke and Hydrogen peroxide
Zheng <sup>210</sup>	SPRR1A	Respiratory system	mice	Carbon monoxide
Zimmermann <sup>211</sup>	SPRR2A,2B	Respiratory system	mice	Different allergens
Chen <sup>22</sup>	SPRR2F,2I	Urinary system	mice	Kidney stone diseases
Saban <sup>156</sup>	SPRR2G	Urinary system	mice	Bacillus calmette-guerin treatment

References corresponding to meta-analysis of SPRR expression presented in Figure 4B Chapter III.

Since then, SPRR expression appeared in all major tissues and cell types, mainly after stress or injury (Table 2). For example, in the lungs SPRR2A and SPRR2B were significantly increased by different allergens in asthmatic mouse models<sup>211</sup>. Also treatments with other stressors resulted in similar expression changes in the lungs. Exposure to tobacco smoke as well as injury induced by reactive oxygen species (ROS) in the form of hydrogen peroxide induces SPRR1B along with genes known to be involved in the reduction of oxidative stress<sup>205</sup>. ROS are also generated following ischemia-reperfusion regimens and result in tissue damage of the heart. Pradervand and co-workers identified massive induction of SPRR1A and SPRR2A after ischemic stress leading to the protection of cardiomyocytes<sup>140</sup>. Protection of the liver against bile, one of the most toxic biological fluids, is provided by the epithelial cells in the biliary tree. Disruption of this barrier by bile duct ligation resulted in a dramatic increase of SPRR proteins and subsequent adaptation of the biliary barrier<sup>133</sup>. In the uterus, SPRR2A was observed as the most upregulated gene during specific stages of the oestrous cycle<sup>182</sup>. During the pro-oestrous and oestrous stages SPRR proteins were highly induced, while at the metoestrous and dioestrus stages they were suppressed again<sup>182</sup>. Diverse SPRR proteins were also highly expressed during the development of the prostate gland<sup>150</sup> and the mammary gland<sup>128</sup>.

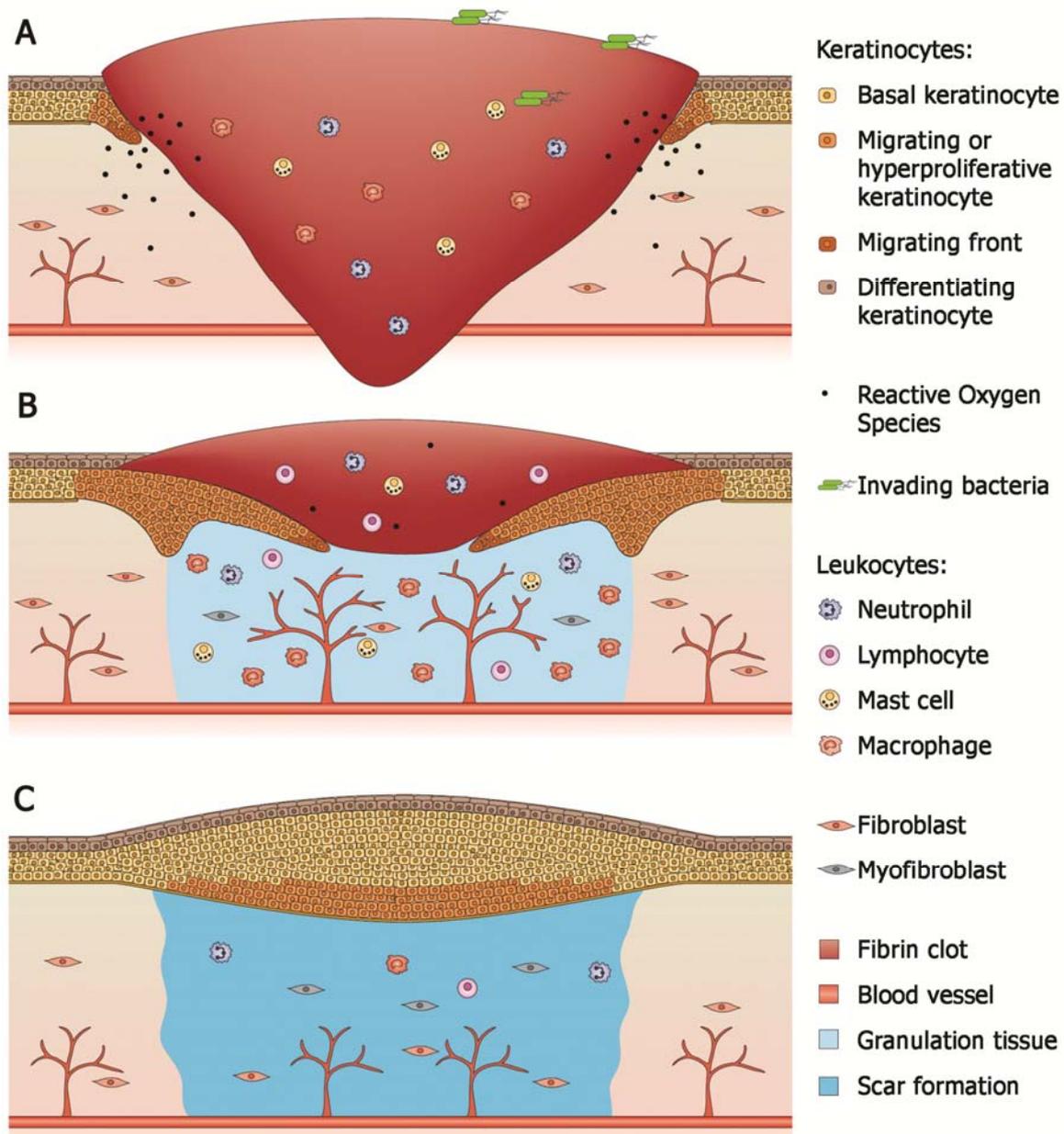
SPRR expression has also been identified in response to neuronal damage<sup>13</sup>. SPRR1A, which was undetectable in uninjured neurons, was the highest induced protein after peripheral axonal damage and was subsequently localised specifically to the injured axons. The axonal outgrowth of these damaged neurons seems to rely on the presence of SPRR proteins, since outgrowth was restricted after downregulation of SPRR1A by siRNA<sup>13</sup>. In addition to tissue damage, stroke induces sequential waves of neuronal growth-promoting genes to activate the process of axonal sprouting. By using microarray at different time-points after stroke, SPRR1 was identified as a novel early responsive gene in peri-infarct cortex regulating the axonal sprouting process<sup>21</sup>. Overall, these expression profiles mainly show upregulation of SPRR in response to a variety of stressors or during the regeneration process after tissue-injury.

### **ROS regulated wound healing**

All wounds, arisen by burning, scratching, myocardial infarction, or any other type of damage, heal in a similar fashion<sup>64</sup>. Following tissue-injury, the surrounding cells react rapidly to allow repair, avoid infections and protect against further loss of blood or tissue<sup>119</sup>. Due to their high accessibility cutaneous wounds are most studied. After disruption of the skin's barrier, a dynamic multistep process of wound healing is activated involving the collaborative efforts of multiple celltypes<sup>117,168</sup>.

As earliest danger signal a tissue-scale gradient of ROS ( $H_2O_2$ ) is produced to attract leukocytes<sup>131</sup>. On top, these produced ROS directly protect the wounded tissue as chemical steriliser against invading microorganisms<sup>118</sup>. Meanwhile, haemostasis is achieved to prevent further blood and fluid loss and a fibrin clot is created as scaffold for the newly formed tissue<sup>64,168</sup>. At a transcriptional level many early response genes are produced to modulate cell behaviour<sup>27</sup>. These are mainly transcription factors needed to produce sufficient amount

of effector genes. The activity of some early response genes (e.g. Nrf2, NF- $\kappa$ B, and AP-1) has been shown to be directly regulated by the amount of ROS present<sup>201,202</sup>. Since the wounded area is still highly oxidised it requires detoxification in order to allow migration and proliferation of the surrounding cells<sup>9</sup>. Subsequent induction of around 200 effector genes has been described at this stage of the wound healing process. The function of these genes ranges from direct antioxidants to extracellular matrix proteins and tissue remodellers<sup>27</sup>. The above described processes all happen within a few hours after wounding.



**Figure 4:** Different stages of cutaneous wound healing. A, During stage one (inflammation), reactive oxygen species (ROS) are generated as chemical steriliser against invading bacteria and as signalling molecules to attract leukocytes. B, During stage two (new tissue formation), keratinocytes migrate into the wounded area to close the gap. C, During stage three (tissue remodelling), wound re-epithelialization is completed and all injury activated processes are terminated. As a result a scar is formed. (Adapted from Schäfer and Werner, 2008<sup>161</sup>).

## Chapter I

During the second stage of wound healing, basal and suprabasal keratinocytes from the innermost epidermal layer start to migrate over the injured dermis<sup>64</sup>. Mechanistically, these migrating keratinocytes are very similar to the collective cell migration during embryonic development and cancer metastasis<sup>55,119</sup>. These diverse migrating cells all show collective polarisation, contain almost identical cytoskeletal machineries and invade a new environment. However, during the well regulated processes of morphogenesis and regeneration, specific cytoprotective genes are expressed to serve as flexible barrier<sup>9,115</sup>. This barrier, at the leading edge of the migrating cells, provides protection to both the migrating keratinocytes themselves as well as the tissue behind. The basal keratinocytes following the actively migrating cells begin to proliferate to eventually restore the epidermal barrier<sup>64,168</sup>.

In the following chapters the role of SPRR proteins in global wound healing is elucidated and the effect of ROS on their protective function will be highlighted. During wound healing, SPRR proteins reduce the high levels of ROS via their cysteine residues. This activity is directly related to their ability to promote cell migration. Likely, SPRR proteins provide all tissues with an efficient, finely tuneable antioxidant barrier, specifically adapted to the tissue involved and the damage inflicted.

# Chapter II

*Distinct functional interactions of human Skn-1 isoforms with Ese-1 during keratinocyte terminal differentiation.*

Adriana Cabral, David F. Fischer, Wilbert P. Vermeij and Claude Backendorf

*J. Biol. Chem.* 2003; 278 (20): 17792-17799

### Abstract

Among the three major POU proteins expressed in human skin, Oct-1, Tst-1/Oct-6 and Skn-1/Oct-11, only the latter induced *SPRR2A*, a marker of keratinocyte terminal differentiation. In this study, we have identified three Skn-1 isoforms, which encode proteins with various N-termini, generated by alternative promoter usage. These isotypes showed distinct expression patterns in various skin samples, internal squamous epithelia and cultured human keratinocytes. Skn-1a and Skn-1d1 bound the *SPRR2A* octamer site with comparable affinity and functioned as transcriptional activators. Skn-1d2 did not affect *SPRR2A* expression. Skn-1a, the largest protein, functionally cooperated with Ese-1/Elf-3, an epithelial-specific transcription factor, previously implicated in *SPRR2A* induction. This cooperativity, which depended on an N-terminal pointed-like domain in Skn-1a, was not found for Skn-1d1. Actually, Skn-1d1 counteracted the cooperativity between Skn-1a and Ese-1. Apparently, the human *Skn-1* locus encodes multifunctional protein isotypes, subjected to biochemical cross-talk, which are likely to play a major role in the fine-tuning of keratinocyte terminal differentiation.

## Introduction

The epidermis constitutes the interface between the organism and the environment and provides protection against physical, chemical and microbial damage. The major epidermal cell type, the keratinocyte, engages in a tightly regulated process of terminal differentiation, which is essential for the protective barrier of the skin and is reflected *in vivo* by the multi-layered structure of the epidermis. The innermost layer (*stratum basale*), connected to the dermis, comprises undifferentiated keratinocytes with a high proliferative potential. The cells committed to terminal differentiation migrate outwards into the non-dividing suprabasal layers, and undergo distinct morphological and structural changes (reviewed in <sup>70</sup>). The transition from proliferating basal keratinocytes to terminally differentiated cells is accompanied by a significant alteration in the gene expression program. The repression of genes required for cellular growth contrasts with the induction of genes related to cell-death and cornification. Aberrations in this tightly choreographed process will affect the expression of epidermal structural proteins, such as those involved in the formation of cytoskeleton, desmosomes and cornified cell envelopes. As a matter of fact many genetic and acquired human dermatoses have been linked to mutations or aberrant expression of these proteins<sup>29,141</sup>.

Whereas the importance of structural proteins in safeguarding the integrity of epidermis and internal squamous epithelia is becoming well understood, little is yet known about the regulatory processes that are involved. Although ubiquitous transcription factors contribute to keratinocyte-specific gene expression<sup>38,52,56,78</sup>, the complex balance between proliferation, stratification and cornification is likely to be coordinated by cell type-specific proteins. Good candidates for such a function are the POU domain transcription factors, a family of more than 40 homeodomain-containing proteins involved in cell differentiation and tissue specification<sup>155,199</sup>. The characteristic POU domain consists of 2 conserved regions, a POU-specific domain and a POU homeodomain, connected by a hypervariable linker region. The entire POU domain is required for DNA binding. The octamer 5'-ATGCAAAT-3' is the most frequent target for POU domain proteins<sup>200</sup>.

Major POU domain factors in skin are Oct-1, Tst-1/Oct-6 and Skn-1/Oct-11<sup>4</sup>. The ubiquitous Oct-1 is expressed in both proliferating and differentiating epidermal keratinocytes, whereas Oct-6 and Skn-1 are primarily expressed in suprabasal layers. Skn-1 is selectively expressed in the epidermis<sup>6,7,62,207</sup>. *In vivo* ablation of murine *Skn-1* did not reveal a specific function for this gene, mainly due to redundancy with Oct-6<sup>7</sup>. Recently, however, the use of *in vitro* raft cultures, disclosed a regulatory role of Skn-1 in keratinocyte proliferation and differentiation<sup>67</sup>.

A further degree of regulatory complexity is due to the fact that at least several POU genes give rise to various isoforms, with specific functional properties and expression patterns (e.g. *Oct-1*, *Oct-2*, *Brn-3* and *Pit-1*)<sup>155</sup>. Also the rat *Skn-1* gene was shown to generate two functionally distinct transcripts, *Skn-1a* and *Skn-1i*<sup>6</sup>. Here we show that the human homologue expresses three isoforms that differentially affected the expression of the *SPRR2A* cornified envelope precursor gene, a marker of keratinocyte terminal differentiation,

whose regulation has been extensively studied<sup>52,109</sup>. This isotype-specific selectivity in *SPRR2A* regulation, which varied from activation to repression, depended on the differential interaction of the Skn-1 isoforms with the epithelium-specific Ets factor Ese-1<sup>134</sup>.

## Experimental Procedures

### Screening of a keratinocyte cDNA library

A human keratinocyte cDNA library, constructed in Lambda ZAP II (Stratagene)<sup>113</sup>, was screened with probes from the POU domains of Oct-1 and Oct-2<sup>25,178</sup> at a stringent hybridization temperature. This yielded among others two independent Skn-1 clones. Plasmid DNA was isolated by *in vivo* excision with Exassist M13 helper phage (Stratagene).

### 5' rapid amplification of cDNA ends (RACE)

5' RACE was performed on poly(A) RNA isolated from cultured normal human keratinocytes, essentially according to a previously described method<sup>42</sup>. Briefly, cDNA synthesized from 1 µg of poly(A) RNA, primed with a 5' biotinylated antisense oligonucleotide specific for Skn-1 (5'-biotin-GAAACCTCTTCTCCAGAGTCAGGCGG), was purified on Dynabeads coated with streptavidine (Dynal) and ligated to a 5' phosphorylated and 3' 3-amino-2-propanol-ether blocked RACE-anchor (5'-phosphate-GCGGCCGCGTCGTGACTGGGAA AACCCOCH<sub>2</sub>CHOHCH<sub>2</sub>NH<sub>2</sub>). PCR, primed with various reverse Skn-1 primers (1: 5'-ACCAAATACTTCACTGAGGCTGGGGTAGGAG; 2: 5'-AACCGCCGAGCCCCACATCTCCCT GT; 3: 5'-GAGGAGACCGCTTTGTTGCTGTGGA; position in GenBank<sup>TM</sup> accession number AF133895: 814, 631 and 394 respectively) and a RACE-primer complementary to the anchor (5'-GGGTTTTCCAGTCACGACGCGGC), was performed with a 2.5:1 mixture of *Pwo* polymerase (Roche Applied Science) and *Taq* polymerase (HT Biotechnology Ltd.) for 40 cycles (20" 94°C, 30" 50-60°C, 2' 72°C). Fragments were cloned in pBluescript II SK(-) (Stratagene).

### Inverse PCR

Genomic DNA was isolated from either simian COS-1 cells or mouse 3T3 fibroblasts by proteinase K digestion and phenol extraction. One µg of DNA was digested with either *Bst*YI or *Sau*3AI and ligated with T4 DNA ligase (Amersham Biosciences). Ligated DNA was used in a PCR reaction with primers designed to contain restriction sites at the 5' end to facilitate subsequent cloning (simian sense primer: 5'-CGAATTTCCACAGACTGG GCCGGGACT; mouse sense primer: 5'-AGAATTTCCACAGACAGGGCCTGGCCT derived from the mouse cDNA sequence (GenBank<sup>TM</sup> accession number Z18537); common antisense primer: 5'-AGAAAGCTTTGTTGCTGTGGAAAGG).

### Semi-quantitative RT-PCR and RNA blotting

Trizol reagent (Invitrogen) was used to isolate total epidermal RNA from tissue obtained either after breast reduction or circumcision. Total RNA (200 ng) was reverse transcribed with Super-RT (SphaeroQ) and random hexamers (Amersham Biosciences). Semi-quantitative PCR was performed according to a previously published procedure<sup>18</sup> but using AmpliTaq Gold (Roche). The following isotype-specific Skn-1 primers were used: Skn-1a: sense: 5'-CACAGATATCAAGATGAGTG, antisense: 5'-TCTGAGATAGCAGGAAGCTG; Skn-1d1: sense: 5'-GTTGTAGCACATGTGTTTCA, antisense: 5'-GAAACCTCTTCTCCAGAGTCAG; Skn-1d2: sense: 5'-TCACCTTAGAGGGAGGAGA, antisense: 5'-CAGCCGGGAGTTGTAGAC. This analysis resulted in products of 330, 581 and 590 basepairs for Skn-1a, Skn-1d1 and Skn-1d2, respectively. Other primers were: SPRR2A: sense: 5'-TGGTACCTGAGCATCGATCTGCC, antisense: 5'-CCAAATATCCTTATCCTTTCTTGG<sup>18</sup>; Ese-1: sense: 5'-CTGAGCAAAGAGTACTGGGACTGTC; antisense: 5'-CCATAGTTGGGCCACAGCCTCGGAGC. RT-PCR conditions for GAPDH were described previously<sup>17</sup>. All primers bridged introns, thus allowing a control for DNA contamination. PCR products for the various Skn-1 isoforms were analyzed on a blot with a probe covering most of the cDNA (generated with the Skn-1a sense and the Skn-1d2 antisense primers). Sequence analysis was used to verify the identity of the various PCR products obtained. SPRR2A RT-PCR products were identified with an SPRR2 cDNA probe<sup>60</sup>. Ese-1 and GAPDH products were detected by ethidium bromide staining. RNA dot-blots were probed with a 511 bp KpnI/EcoRV fragment (3' UTR) of Skn-1 (GenBank<sup>TM</sup> accession number AF133895).

### In situ hybridization

Experiments were carried out as described previously<sup>17</sup>. Digoxigenin-labeled (Roche Applied Science) sense and antisense RNA probes were generated using the 511 bp KpnI/EcoRV fragment (3' UTR) of Skn-1 described above, a 680 bp fragment of SPRR2<sup>69</sup> and 475 bp XhoI/BglII fragment (3'UTR) of Ese-1 (GenBank<sup>TM</sup> accession number U73844).

### Expression plasmids

Protein expression plasmids were constructed by introducing the *Skn-1* coding sequences in the HindIII and EcoRI sites of the T7 expression plasmid pT7-2. Plasmids encoding the whole open reading frame were generated for *Skn-1a* (pPOU117, from exon 1-13), *Skn-1d1* (pPOU123, from codon A in intron 5 to exon 13) and *Skn-1d2* (pPOU121, exon 8-13). For *Skn-1d1*, 3 mutants were constructed either by site-directed mutagenesis (point mutations in pPOU124 and pPOU118) or by PCR (pPOU137). Proteins encoded by these plasmids were synthesized in a coupled *in vitro* transcription-translation system (TnT reticulocyte lysate, Promega) in the presence of <sup>35</sup>S-methionine (Amersham). Full-length *Ets-2*, *Ese-1*, and *Oct-2* cDNAs were isolated by screening the above mentioned human keratinocyte cDNA library either with Ets-specific<sup>159</sup> or Oct-1/2 POU domain probes. For transfection the different cDNAs were cloned into the RSV-H20 expression plasmid<sup>158</sup>. Expression plasmids for Oct-1 and Oct-6 were gifts of Dr. W. Herr (Cold Spring Harbor) and Dr. D. Meijer (Erasmus University Rotterdam), respectively.

### Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were performed as described in Fischer *et al*<sup>52</sup>. The *SPRR2A* octamer oligonucleotide (GGATAAATTTGCATCTGGCT) was labeled with T4 polynucleotide kinase, purified by denaturing gel electrophoresis and reverse-phase chromatography, and subsequently annealed to the unlabeled complementary strand. In each reaction 2  $\mu$ l of programmed reticulocyte lysate and 20 fmol of labeled oligonucleotide duplex were used.

### Cell culture, transient transfections, CAT and luciferase assays.

HaCaT cells were grown in DMEM with 10% bovine calf serum (Hyclone). Confluent cultures were transfected by incubating 5 cm culture dishes for 2 h with 5  $\mu$ g of reporter plasmid (CAT or luciferase), 2.0  $\mu$ g of Rous sarcoma virus expression plasmids (including compensating amounts of empty Rous sarcoma virus vector) and 40  $\mu$ g of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium salts transfection reagent. Monolayers were washed with PBS and incubated for 24 h in culture medium. CAT assays were performed as described<sup>52</sup>. Luciferase activity was measured with the Luciferase Assay System (Promega) essentially as previously described<sup>158</sup>. All transfections were performed at least in triplicate. The *SPRR2A* minimal promoter-driven CAT plasmid pSG55 has been previously described<sup>52</sup>. Luciferase plasmids were constructed in pGL3 (Promega) and contained the following *SPRR2A* promoter inserts all derived from previously described plasmids<sup>52</sup>: pSG350-wt (minimal promoter): pSG55; pSG390-Ets mutant: pSG212; pSG527-octamer mutant: pSG185.

## Results

### Structural characterization and organization of Skn-1 isoforms

Searching the human genome database at NCBI with the previously described human *Skn-1a* cDNA (GenBank<sup>TM</sup> accession number AF133895) disclosed one sequence (GenBank<sup>TM</sup> accession number AP001150), encompassing the complete human gene. The 2868 bp cDNA sequence comprises 13 exons and extends over a 70-kilobase genomic region (Fig. 1A). The characteristic POU domain is encoded by exons 7-10.

The screening of a human keratinocyte cDNA library with a POU domain probe and 5' RACE (see Experimental Procedures) identified 3 Skn-1 isoforms, namely the previously described Skn-1a<sup>66</sup> and two novel variants, Skn-1d1 and Skn-1d2. Comparison with the genomic sequence revealed that the 5' end of *Skn-1d1* and *Skn-1d2* corresponded to sequences in introns 5 and 7, respectively (Fig. 1A). The absence of other introns in these transcripts confirmed the mRNA origin of both clones.



The sequence of the *Skn-1d2* transcript, which initiates in intron 7, revealed 3 intron-encoded AUG codons, in frame with the POU domain sequence, but followed by a termination codon at position 154 (Fig. 1C). Potential start codons for open reading frames are found in exon 8 (codon D and/or codon E).

### DNA binding activity of the hSkn-1 variants

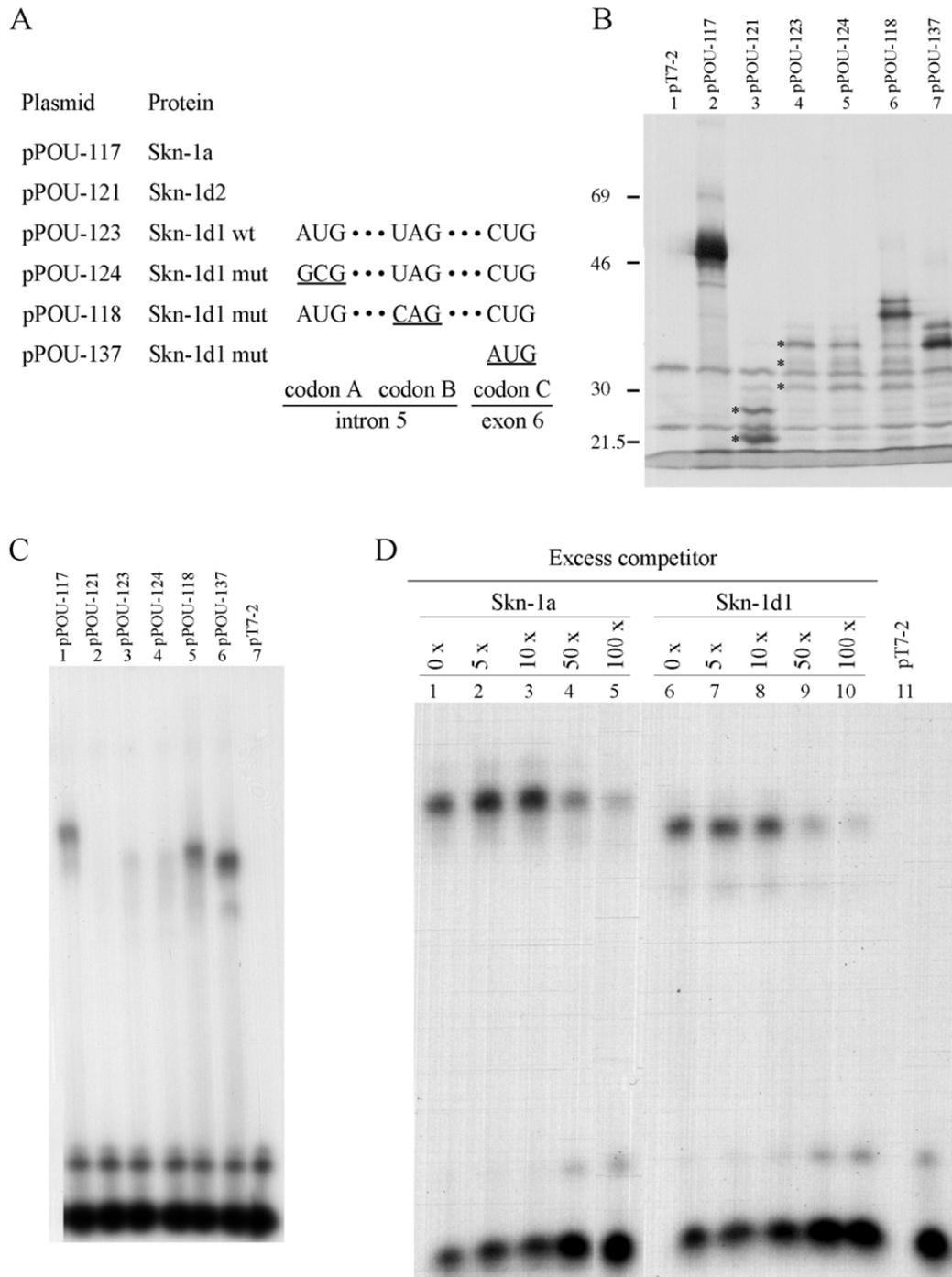
The integrity of the Skn-1 expression plasmids and their coding potential were verified by producing proteins *in vitro* with the TNT reticulocyte lysate system (Promega) (Fig. 2B). Skn-1a (pPOU-117) migrated at the predicted molecular mass (47.5 kDa) and bound to the *SPRR2A* octamer site (Fig. 2C). The Skn-1d2 isoform (pPOU-121) generated two different products (Fig. 2B). The slow migrating product has an apparent molecular mass of 25 kDa, which is in accordance with a protein initiating at codon D in the POU-specific domain (Fig. 1C). The 21.5 kDa product corresponds to a protein starting at initiation codon E. None of these Skn-1d2 isoforms is capable of binding to the *SPRR2A* octamer site (Fig. 2C), which is likely due to the partial deletion of the POU-specific domain (Fig. 1A).

The wild type *Skn-1d1* transcript (plasmid pPOU-123) codes for a major polypeptide of 35 kDa, and two minor products of 33 and 31 kDa (Fig. 2B). To investigate translational initiation of Skn-1d1 proteins more precisely, constructs with mutations in codons A, B or C (Fig. 1B and 2A) were generated. Fig. 2B shows that translation of the major Skn-1d1 polypeptide most likely initiates at codon C (a weak CUG initiation codon). Indeed, pPOU-137, in which this codon was changed into an efficient AUG initiator, yielded high levels of a product of 35 kDa, identical to the largest polypeptide from the wild type transcript. This cDNA was used in transfection experiments. The minor 33 and 31 kDa proteins were not investigated further.

Electrophoretic mobility shift assays were performed to compare the binding affinity of Skn-1a (pPOU-117) and Skn-1d1 (pPOU-137) to the *SPRR2A* octamer site. Labeled double-stranded *SPRR2A* octamer oligonucleotide was incubated with reticulocyte lysate programmed with either Skn-1a or Skn-1d1. As shown in Fig. 2D, competition with increasing amounts of unlabeled binding site revealed that both Skn-1 variants bound the *SPRR2A* octamer site with similar affinities.

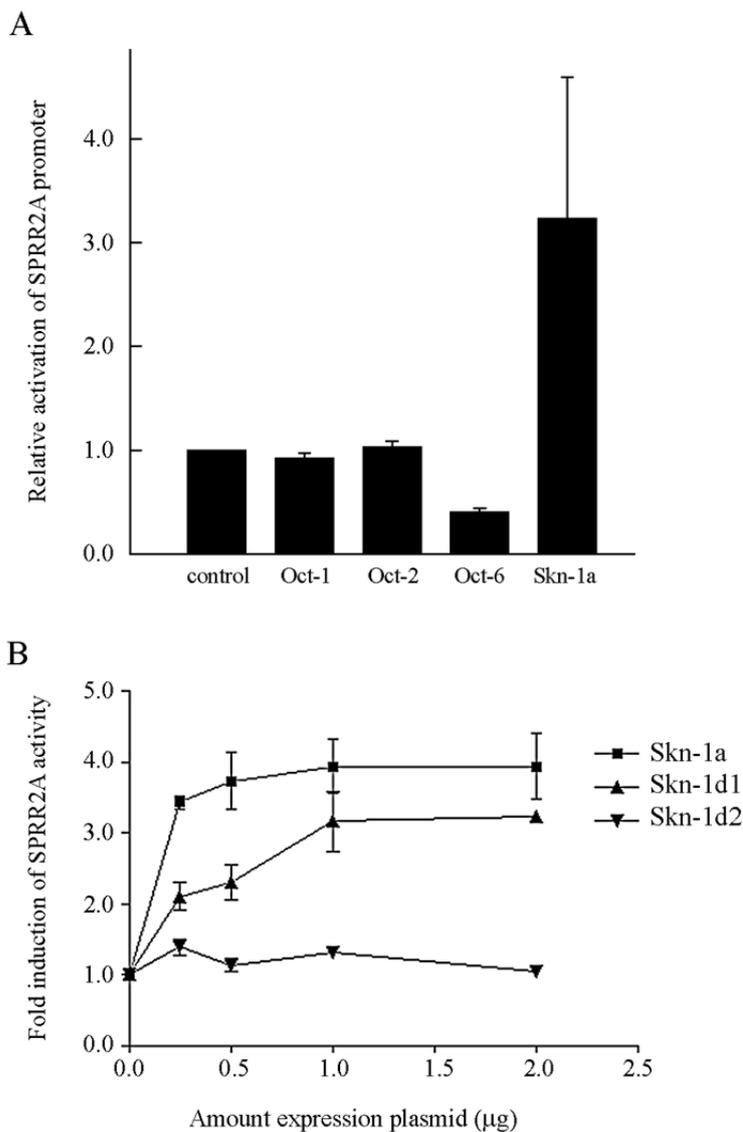
### Transactivation potential of Skn-1 isoforms

In Fig. 3A, the *SPRR2A* proximal promoter, fused to the CAT reporter and encompassing all cis-elements necessary for expression during keratinocyte terminal differentiation<sup>52</sup>, was transiently transfected into HaCaT cells, which contain low levels of endogenous POU proteins<sup>67</sup>. We first evaluated the transactivation potential of Oct-1, Oct-6/Tst-1 and Skn-1a, the major POU domain proteins expressed in skin<sup>4</sup>, and of Oct-2, a lymphoid specific transcription factor<sup>25</sup>, also expressed in cultured human keratinocytes<sup>7</sup> (our unpublished observation). Oct-1 and Oct-2 did not affect baseline expression of *SPRR2A*, Oct-6 repressed promoter activity by ~70% and Skn-1a was the only POU domain protein tested that mediated gene activation (3-4 fold induction, Fig. 3A).



**Fig. 2.** DNA binding activity of *in vitro* synthesized Skn-1 isotypes. **A**, Plasmids used for *in vitro* transcription-translation. The various mutations in *Skn-1d1* are indicated. Nomenclature of codons A, B and C is according to Fig. 1B. **B**, SDS-PAGE analysis of *in vitro* synthesized Skn-1 proteins. Reticulocyte lysates were programmed with the indicated *Skn-1* plasmid or with a control plasmid (pT7-2, lane 1). Molecular weight markers (Amersham Biosciences) are indicated in the margin (in kDa). Skn-1d1 and Skn-1d2 specific products are marked with asterisks. **C**, Electrophoretic mobility shift assay of Skn-1 programmed lysates (lanes 1-6), or un-programmed lysate (lane 7) with the *SPRR2A* octamer site. **D**, Affinity of Skn-1a or Skn-1d1 for octamer binding: reticulocyte lysate containing identical amounts of either Skn-1a or Skn-1d1 protein were incubated with 20 fmol of the <sup>32</sup>P-labeled *SPRR2A* octamer site and competed with the indicated molar excess of unlabeled binding site.

To investigate the relative contribution of the individual Skn-1 isoforms in the regulation of the *SPRR2A* gene, the transactivation potential of the three variants was determined by transfecting increasing amounts of isotype-specific expression plasmids into HaCaT cells, programmed with an *SPRR2A*-luciferase construct. Both Skn-1a and Skn-1d1 up-regulated the *SPRR2A* promoter by 3-4 fold. The saturation kinetics were, however, different because lower amounts of Skn-1a were needed to reach a plateau, indicating that Skn-1a can transactivate the *SPRR2A* promoter more efficiently than Skn-1d1 (Fig. 3B). Skn-1d2 had no effect on *SPRR2A* promoter activity, even at higher doses. Collectively, these results indicate that among several POU domain proteins only Skn-1a and Skn-1d1 are able to activate the *SPRR2A* promoter in an *in vitro* transient transfection experiment. Hence, it was important to investigate whether a similar direct relation existed also *in vivo* between Skn-1a/d1 and *SPRR2A* expression.



**Fig. 3.** Effect of various POU proteins on *SPRR2A* promoter activity. A, The *SPRR2A*-CAT reporter construct (pSG55) was either transfected alone or together with expression plasmids for Oct-1, Oct-2, Oct-6 and Skn-1a into HaCaT keratinocytes as described under "Experimental Procedures". CAT activity was determined 24 h after transfection as previously described<sup>52</sup> and was related to the basal activity of pSG55. B, Increasing amounts of Skn-1a, Skn-1d1 and Skn-1d2 expression plasmids were co-transfected with the *SPRR2A*-luciferase reporter construct (pSG350) and luciferase activity was determined 24 h later essentially as previously described<sup>158</sup>.

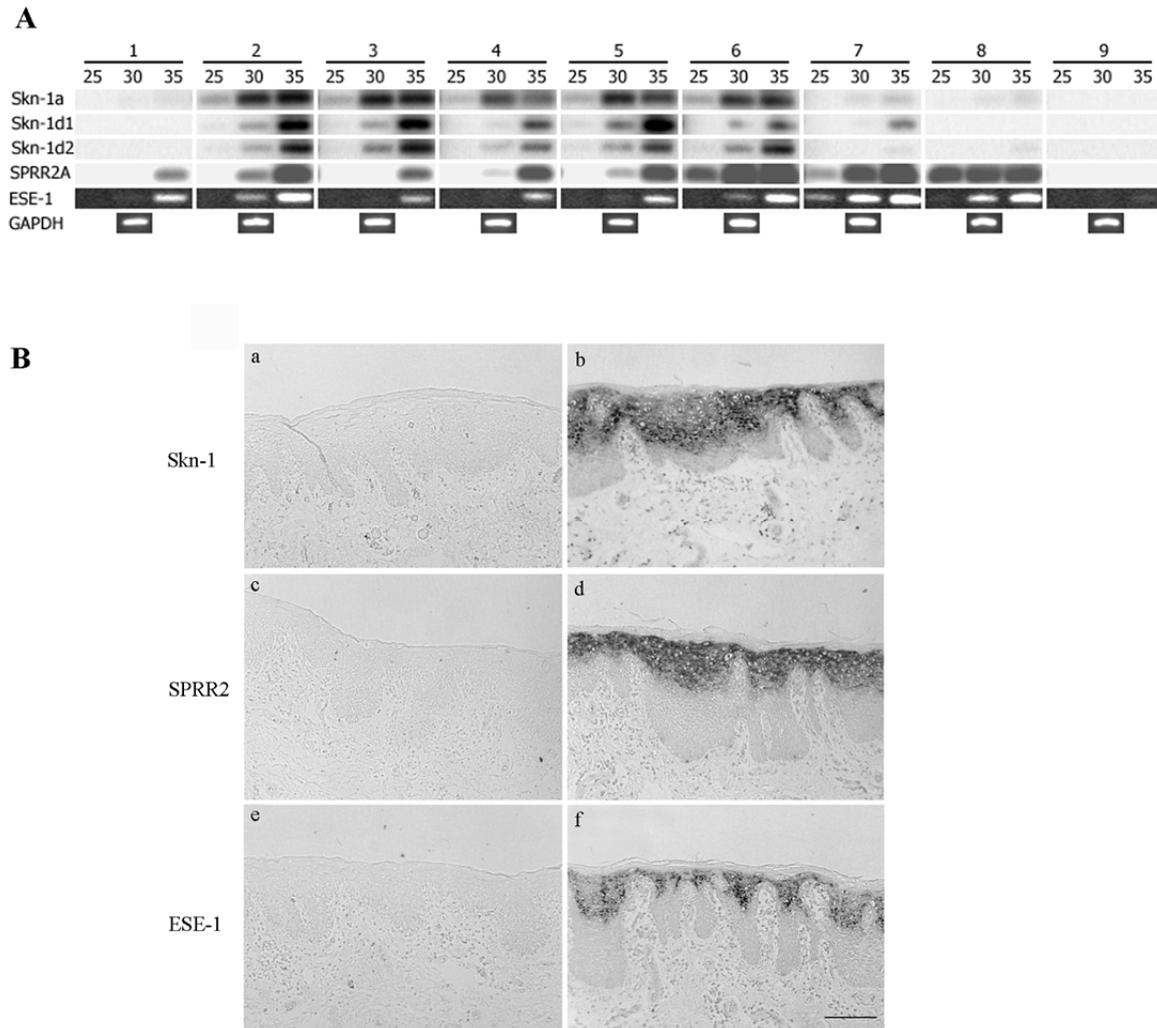
**Correlation between *SPRR2A* and *Skn-1a* expression *in vivo*.**

Initially, a panel of 50 different RNA samples from various human tissues was analyzed with a *Skn-1* specific probe (3' UTR) which detects all isoforms. *Skn-1* expression was restricted to a few stratified squamous epithelia including epidermis, cervix and foreskin (results not shown). To compare the expression pattern of the 3 human *Skn-1* variants with *SPRR2A* expression, semi-quantitative RT-PCR with isotype-specific primer sets was performed on RNA isolated from either total skin, epidermis, foreskin, cervix or cultured primary keratinocytes. RNA from uterus, which does not contain *SPRR2A* transcripts<sup>18</sup>, was used as a negative control. The results in Fig. 4A show that *Skn-1a* was expressed in all squamous epithelia (lanes 1-6 and 8) and in cultured keratinocytes (lane 7), although at different levels. Expression of *Skn-1d1* was more heterogenous, as it was not detected in the skin and cervix samples from lanes 1 and 8, respectively. Relatively high expression levels of *Skn-1d1* (already visible after 25 cycles) were found in the epidermal RNAs from lanes 2, 3 and 5. *Skn-1d2* expression was, in general, similar to the one of *Skn-1a*. The absence of *Skn-1* transcripts in uterus correlated well with the absence of *SPRR2A* expression. However, no clear correlation was found in the other samples between the expression level of one of the *Skn-1* isotypes and *SPRR2A* expression, indicating the involvement of other transcription factors. Indeed, previous work from our laboratory has shown that expression of *SPRR2A* relied on interdependent regulatory promoter elements, recognized by various classes of transcription factors<sup>52</sup>. Consequently, we have also monitored the expression of the epithelial specific Ets factor Ese-1, previously implicated in *SPRR* regulation<sup>134,159</sup>. Expression of this gene was found in all samples, including uterus (although at low levels). The highest levels were found in the epidermal sample of lane 2, in cultured keratinocytes (lane 7) and in cervix (lane 8). Again, no strict correlation was found between the levels of *Ese-1* and *SPRR2A* expression (Fig. 4A).

To investigate whether Skn-1 and Ese-1 co-localize *in vivo*, we monitored the stratum-specific expression of *Skn-1*, *Ese-1* and *SPRR2A* in sections of foreskin (Fig. 4B). Whereas *Skn-1* (the 3' UTR probe used detects all isoforms) was present in most suprabasal layers, *Ese-1* expression was confined to the more differentiated layers. Most importantly, the distribution of both factors overlapped with the expression of *SPRR2A*. Because both octamer and Ets binding sites in the promoter are essential for *SPRR2A* expression<sup>52</sup>, we questioned whether Skn-1 isoforms and Ese-1 had the potential to cooperate in *SPRR2A* transactivation.

**Ese-1 selectively cooperates with Skn-1a in the transactivation of the *SPRR2A* promoter.**

HaCat cells were transfected with the *SPRR2A*-luciferase reporter construct (PSG350) together with increasing amounts of the different transcription factors, either alone or in various combinations (Fig. 5). The total amount of transfected expression plasmid was kept constant by compensating with the empty vector. Skn-1a, Skn-1d1 and Ese-1 alone induced the *SPRR2A* promoter 3 to 4 fold at saturation. However, cotransfections of Skn-1a and Ese-1 activated the same promoter in a dose dependent manner up to 8 fold, suggesting functional cooperativity between these two transcription factors (Fig. 5A). Such synergy was



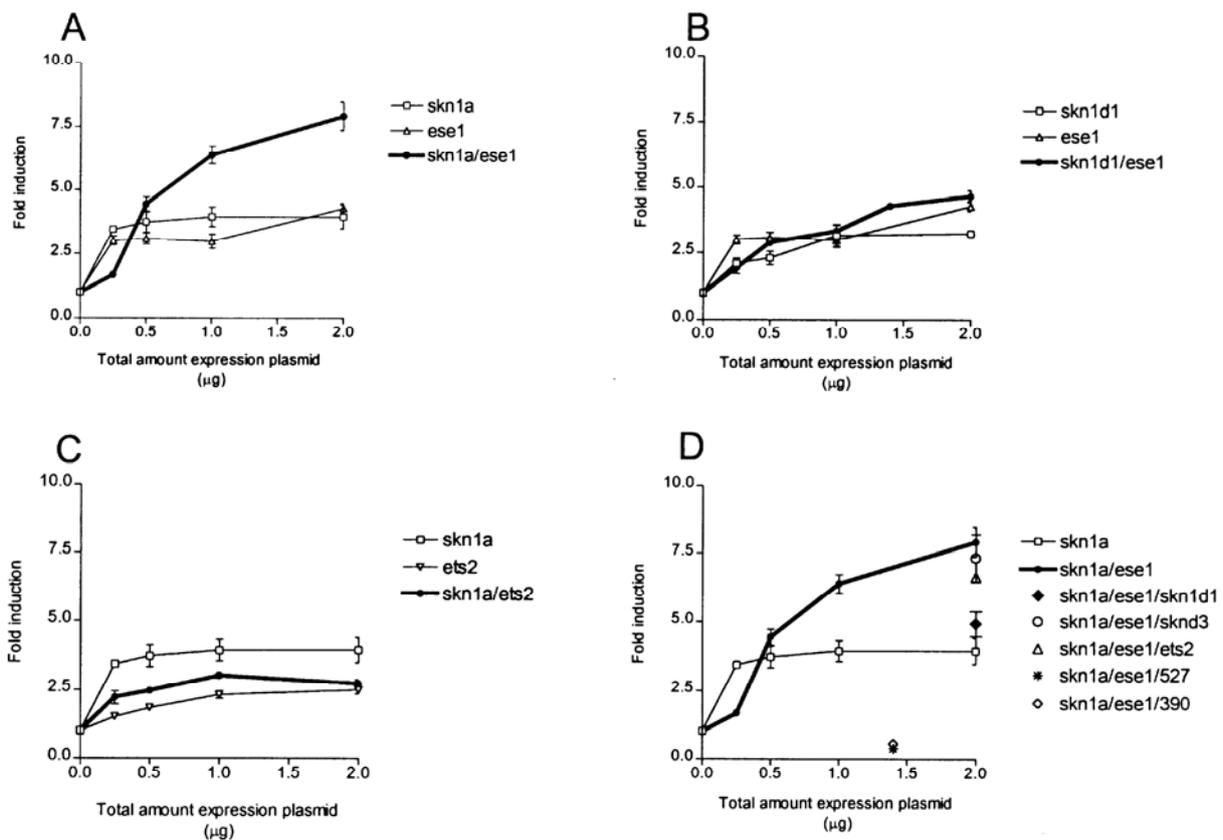
**Fig. 4.** A, Semi-quantitative RT-PCR analysis of *Skn-1*, *Ese-1*, *SPRR2A* and *GAPDH* expression in various human tissues and cells. RNA isolated from total skin (lane 1), isolated epidermis (lanes 2-5), foreskin (lane 6), cultured primary keratinocytes (lane 7), cervix (lane 8) and uterus (lane 9) were analyzed with *SPRR2A*, *Ese-1*, *GAPDH* and isoform-specific *Skn-1* primers. PCR was performed with 25, 30 and 35 cycles and products were either detected with gene-specific probes (*Skn-1* isoforms and *SPRR2A*) or by direct ethidium bromide staining (*Ese-1* and *GAPDH*). For *GAPDH* only the 30 cycle product is shown. B, In situ hybridization performed with digoxigenin-labeled *Skn-1*, *SPRR2* and *Ese-1* specific RNA probes. A foreskin section was analyzed with sense (a, c, e) and antisense (b, d, f) probes. Bar, 100  $\mu$ m.

not found in combinations of Skn-1d1 with Ese-1 (Fig. 5B), nor with Skn-1a and Ets-2 (Fig. 5C), another Ets transcription factor previously implicated in keratinocyte terminal differentiation<sup>103</sup>. Apparently, only Skn-1a can specifically cooperate with Ese-1 in the activation of the *SPRR2A* gene.

Figure 5D shows the effect of Skn-1d1, Skn-1d2 or Ets-2 on the cooperative activation of the *SPRR2A* promoter by Skn-1a and Ese-1. Although neither Skn-1d2 nor Ets-2 was able to affect the cooperativity between Skn-1a and Ese-1, a clear drop in promoter

activity was observed when Skn-1d1 was included. Apparently Skn-1d1 is able to compete with Skn-1a for promoter binding even in the presence of Ese-1, resulting in a complete abrogation of the synergistic effect. In the case of Ets-2 the situation is different: Ets-2 can at least partially down-modulate the activity of Skn-1a in the absence of Ese-1 (Fig. 5C) but not in its presence (Fig. 5D). Furthermore it is shown that mutations in either Ets (PSG390) or octamer binding site (PSG527) resulted in a complete inhibition of *SPRR2A* promoter activity, in agreement with our previously published results<sup>52</sup>.

Taken together, our results show that although both Skn-1a and Skn-1d1 could transactivate the *SPRR2A* promoter, only Skn-1a was able to functionally cooperate with Ese-1, resulting in enhanced transactivation. Skn-1d1 was able to counteract this functional cooperativity.



**Fig. 5.** Transcription factor cooperativity and antagonism during *SPRR2A* gene regulation: Cotransfection of the *SPRR2A*-luciferase reporter construct (5 μg) with the indicated total amounts (μg) of expression plasmids for Skn-1a and/or Ese-1 (A), Skn-1d1 and/or Ese-1 (B), Skn-1a and/or Ets-2 (C). When more than one transcription factor was transfected in the same mixture, the plasmids were always added in equal amounts. The total amount of transfected expression plasmid was kept constant at 2 μg by compensating with empty vector. D, Cotransfection of the wild-type (pSG350) or mutant (octamer site: pSG527; Ets site: pSG390) *SPRR2A* promoter constructs with Skn-1a/Ese-1 and the indicated transcription factor expression plasmids. Transfection and luciferase measurements were as described previously<sup>158</sup>.

## Discussion

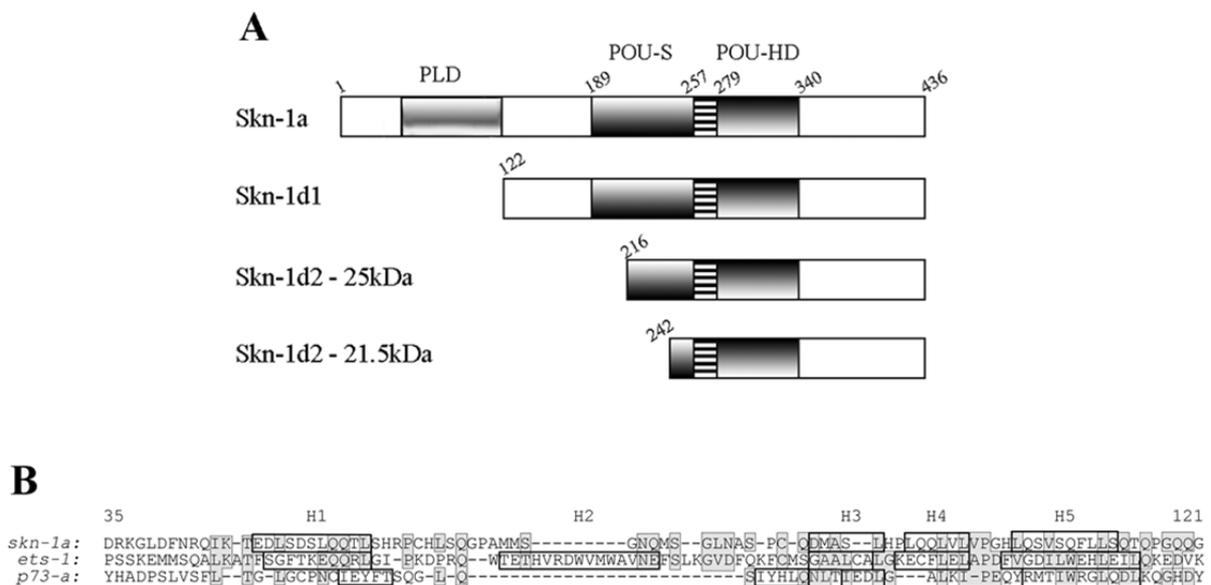
In this study, we have identified and characterized three isoforms of the human *Skn-1/Oct-11* gene and assessed their ability to regulate the human *SPRR2A* gene. The promoter of this gene has been well characterized in the past and its activation during keratinocyte differentiation has been well documented. It encompasses an octamer binding site, that is recognized by Skn-1 and is essential for promoter activity together with three other transcriptional control elements, bound by respectively the Ets, Irf and Klf transcription factor families<sup>52</sup>. Among these, only the Ets binding activity has previously been identified as the epithelial-specific Ets factor, Ese-1<sup>134</sup>. Our previous finding, that destruction of a single binding site results in a complete loss of promoter activity, has stressed the importance of signal integration and transcription factor cooperativity in the regulation of this gene<sup>52</sup>. Here, we have used this well-documented facet of *SPRR2A* regulation, to analyze the regulatory abilities, including possible synergistic/antagonistic activities, of the three different Skn-1 isoforms that we have identified.

The human *Skn-1* gene produces three mRNA species, encoding proteins with various N-termini (Fig. 6A). The two shorter mRNA variants are the result of internal promoter usage and initiate within introns 5 and 7. The observation, that the three transcripts are differentially expressed in various skin samples and in cultured human keratinocytes (Fig. 4A), indicates that the corresponding promoters function independently and are subjected to selective regulation. Although the Skn-1d1 and Skn-1d2 variants originate within introns, termination codons prevent the addition of specific N-terminal ("intron" encoded) sequences to the proteins. Consequently, Skn-1d1 and Skn-1d2 can be viewed as N-terminal deletions of Skn-1a.

*Skn-1a*, which encodes the full-length protein, is identical to the mRNA previously described by others<sup>66</sup>, and is highly homologous to its rat and mouse orthologues<sup>6,62</sup>. *Skn-1a* transcripts are expressed in all human skin samples analyzed (Fig. 4A). The higher level of expression in epidermal samples (lanes 2-5), as compared with a total skin preparation (lane 1), correlates with the epidermal expression of this gene (Fig. 4B). Our analysis clearly shows that *SPRR2A* expression is not strictly linked with either *Skn-1A* or *Ese-1* expression, but it clearly correlated with the presence of both factors. For instance, low levels of Ese-1 in uterus do not induce *SPRR2A* expression due to the absence of Skn-1a (Fig. 4A, lane 9). However, weak expression of Skn-1a can be compensated by the presence of Ese-1, and result in efficient *SPRR2A* expression in cultured keratinocytes and cervix (lanes 7-8). These *in vivo* expression data are in line with our transient transfection experiments, which have established cooperativity of Skn-1a and Ese-1 in *SPRR2A* promoter transactivation (Fig. 5) and corroborate the previously identified necessity for signal integration in the regulation of *SPRR2A*<sup>52</sup>.

Skn-1d1 did not have the ability to synergize with Ese-1. Because Skn-1a and Skn-1d1 exhibited comparable DNA-binding activities with the *SPRR2A* octamer site, the functional cooperativity between Skn-1a and Ese-1 is likely to be mediated by a specific domain in Skn-1a, which is not present in Skn-1d1. Hence, the 122 aminoacid N-terminal

part of Skn-1a, which is absent in Skn-1d1 (Fig. 6A), was screened for possible structural domains by using 3D-PSSM<sup>90</sup>, a fold recognition program at Imperial Cancer Research Fund (London) (<http://www.sbg.bio.ic.ac.uk/3dpssm>). This search has revealed between amino acids 35-121 a region with significant similarity to the canonical sterile  $\alpha$  motif/pointed domain (Fig. 6B). This fold, which is found in many different proteins, including for instance the Ets and p53 transcription factor families, functions essentially as a protein-protein interaction interface<sup>8,105</sup>. It might seem tempting to speculate that the pointed-like domain (PLD) in Skn-1a interacts directly with the Ets-1 pointed domain. However, such an interaction would not account for the highly specific cooperativity between Skn-1a and Ets-1 that we have observed. Ets-2 also contains a pointed domain; it has the ability to bind to the *SPRR2A* Ets site, but it does not synergize with Skn-1a. Consequently, it seems more likely that the Skn-1a pointed-like domain contacts a protein domain in Ets-1, which is specific for this factor.



**Fig. 6.** A, Schematic representation of the various Skn1 isoforms identified in this study. The POU-specific domain (POU-S), the POU-homeodomain (POU-HD) and the pointed-like domain (PLD) are represented. The amino acid numbering corresponds to the Skn-1a isoform. B, Comparison of the N-terminal region (amino acids 35-121) of Skn-1a with the sterile  $\alpha$  motif /pointed domains of Ets-1 and p73- $\alpha$ . Identical and similar amino acid residues are highlighted in grey and the predicted  $\alpha$ -helices in Skn-1a (3D-PSSM) and those in the resolved structures of Ets-1<sup>169</sup> and p73<sup>196</sup> are boxed. As a reference  $\alpha$ -helices in Ets-1 are numbered H1-H5.

The human *Skn-1d1* mRNA is conserved among rodents and primates; however, its coding potential has clearly changed during evolution. Although in primates the region encoded by intron 5 is not translated (due to in-frame termination codons), it encodes in rat and mouse an N-terminal 32-amino acid domain (Fig. 6A) that is responsible for the inhibitory activity of the Skn-1i isoform<sup>6</sup>. Skn-1d1 did also inhibit *SPRR2A* promoter activity in the presence of Skn-1a and Ets-1 (Fig. 5D), although it had the ability to activate the same promoter when present on its own (Fig. 3). Furthermore, in our *in vivo* analysis, high *Skn-*

*1d1* expression was inversely related to high *SPRR2A* expression. For instance in the epidermal samples of lanes 2, 3 and 5, where high expression of *Ese-1*, *Skn-1a* and *Skn-1d1* is monitored, expression of *SPRR2A* is clearly lower than in foreskin (lane 6), where high expression of *Skn-1a* and *Ese-1* contrasts with low expression of *Skn-1d1*. Similarly, comparison of lanes 7 and 8 (cultured keratinocytes and cervix) reveals that similar levels of *Skn-1a* and *Ese-1* lead to lower expression of *SPRR2A* in the cultured cells (lane 7) due to the presence of *Skn-1d1*. The mechanisms by which hSkn-1d1 and mSkn-1i inhibit gene expression differ, however, fundamentally. Whereas the mSkn-1i inhibitory domain acts in *cis* and inhibits DNA binding<sup>6</sup>, the human counterpart has the same DNA binding affinity as *Skn-1a*. Due to this property it can compete with *Skn-1a* for octamer binding, and interfere in this way with the cooperative activation of *SPRR2A* by *Skn-1a* and *Ese-1*. The differential effect of *Skn-1d1* on *SPRR2A* expression, in the presence or absence of *Skn-1a*/*Ese-1*, is also interesting from a different point of view. It might actually shed light on several unexplained findings, concerning the relative transactivation potential of *Skn1a* and various truncated constructs, including an N-terminal deletion<sup>66,180</sup>. In these experiments the outcome depended greatly on the specific promoter that was tested. Although the N-terminal deletion of *Skn-1a* induced K10 and HPV1a promoter activity, no discernible effect was observed on HPV-18, and K14 was inhibited. Our results suggest that such variable outcomes can be expected and that they are likely to depend mainly on the specific transcription factor occupancy of the promoter that is analyzed. This is especially true for promoters subjected to strict combinatorial gene regulation, such as *SPRR2A*<sup>52</sup> and most likely also for the various promoters mentioned above. Consequently, to be able to fully appreciate the outcome of transcription factor truncation experiments, a reasonable knowledge of the regulatory configuration of the promoter, that is analyzed, is a prerequisite. A similar complexity has also recently been observed for the *profilaggrin* promoter and is discussed by the authors<sup>78</sup>.

The human *Skn-1d2* isoform codes for two proteins, lacking the first 27 or 53 aminoacids of the POU-specific domain, whereas the POU-homeodomain is left intact (Fig. 6A). It has previously been shown that both sub-domains are required for DNA binding<sup>91</sup>. This probably explains why *Skn-1d2* neither bound to the *SPRR2A* octamer site nor affected promoter activity. However, *Skn-1d2* was widely expressed, with transcripts detected in all skin samples analyzed. This feature suggests that *Skn-1d2* might have a physiological role, which does not depend on DNA binding. Indeed, POU domain proteins have been shown to regulate transcription also through protein-protein contacts, in a DNA-binding independent manner. These interactions are often mediated by the POU-specific, the POU-homeo or both domains and target other transcription factors, co-regulators, basal factors and chromatin components (for a recent review, see Ref.<sup>5</sup>). For instance, downregulation of keratin 14 is mediated by the POU-domain of either *Skn-1a* or Oct-6, and does not involve DNA binding to the *K14* promoter<sup>180</sup>. In the case of Oct-1, several protein-protein interactions are mediated solely via the POU homeodomain<sup>5</sup>. Consequently, it is possible that the *Skn-1d2* encoded proteins, which still have intact POU homeodomains, have the ability to affect gene expression by similar protein-protein interactions. More experiments will be needed to unravel a possible regulatory function of the *Skn-1d2* isoforms.

Our work describes for the first time a functional interaction between the *Skn-1a* and *Ese-1* transcription factors. The differential cross-talk of *Skn-1a* and *Skn-1d1* with *Ese-1*

highlights the complexity of combinatorial gene regulation during keratinocyte terminal differentiation. The strict dosage of Skn-1 isoforms is likely to guarantee both the fine-tuning of the process of epidermal maturation and its adaptation to external and environmental hazards.

### Acknowledgements

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# Chapter III

*Skin cornification proteins provide global link between ROS detoxification and cell migration during wound healing.*

### Abstract

Wound healing is a complex dynamic process characterised by a uniform flow of events in nearly all types of tissue damage, from a small skin scratch to myocardial infarction. Reactive oxygen species (ROS) are essential during the healing process at multiple stages, ranging from the initial signal that instigates the immune response, to the triggering of intracellular redox-dependent signalling pathways and the defence against invading bacteria. Excessive ROS in the wound milieu nevertheless impedes new tissue formation. Here we identify small proline-rich (SPRR) proteins as essential players in this latter process, as they directly link ROS detoxification with cell migration. A literature-based meta-analysis revealed their up-regulation in various forms of tissue injury, ranging from heart infarction and commensal-induced gut responses to nerve regeneration and burn injury. Apparently, SPRR proteins have a far more widespread role in wound healing and tissue remodelling than their established function in skin cornification. It is inferred that SPRR proteins provide injured tissue with an efficient, finely tuneable antioxidant barrier specifically adapted to the tissue involved and the damage inflicted. Their recognition as novel cell protective proteins combining ROS detoxification with cell migration will provide new venues to study and manage tissue repair and wound healing at a molecular level.

## Introduction

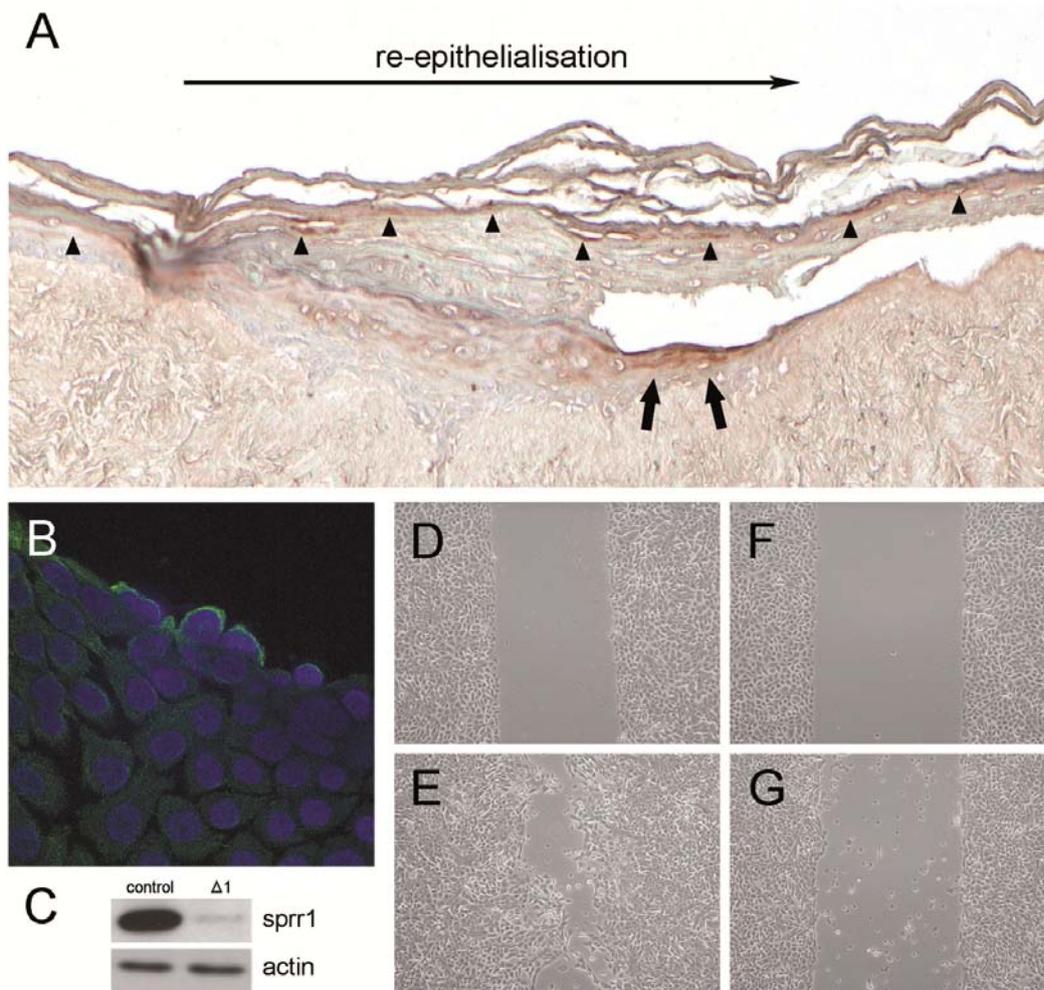
Reactive oxygen species (ROS) are produced directly after tissue damage and are essential during various stages of the healing process, ranging from the initial signal that instigates the immune response to the defence against invading bacteria<sup>131,162</sup>. For efficient healing, the injured tissue has to rapidly adapt via ROS detoxification to allow correct regulation of redox-sensitive signalling pathways implicated in the healing process<sup>162,165,168</sup>. This rapid response is crucial in all tissues especially during injury, notably in the heart and brain<sup>140</sup>. Due to its accessibility, cutaneous wounding, where a temporal defect results in a local loss of the skin's protective barrier function<sup>64,117</sup>, is particularly well studied. This barrier, which normally provides protection against external insults, is maintained, restored and continuously self-renewed by proliferating, migrating and terminally differentiating keratinocytes<sup>19,130</sup>. When basal keratinocytes initiate the differentiation process, they undergo morphological changes and express several cornified envelope precursor proteins, such as involucrin, loricrin, and the small proline-rich (SPRR) proteins, ending as a layer of dead flattened cells on the skin surface. In this upper cornified layer, the precursor proteins are cross-linked at the cell periphery and form together with lipids the cornified cell envelope (CE), a structure which is responsible for the major barrier function of the skin<sup>84,151</sup>. The SPRR gene family is part of the epidermal differentiation complex (EDC) localised on human chromosome 1q21 and mouse chromosome 3. Many of the genes in this locus are coordinately regulated during epidermal differentiation. SPRRs are specifically known as stress-inducible proteins involved in the adaptation of the skin barrier following various forms of stress<sup>18</sup>. During wound healing keratinocytes are the first cells to adapt to defects in the barrier and switch from a differentiating to a migratory mode in order to form new tissue<sup>54,64</sup>. Migrating keratinocytes lack the CE and therefore an alternative protective barrier appears crucial to enter the highly oxidised wound.

Here we show that SPRR proteins protect keratinocytes from excessive ROS by direct quenching via their cysteine residues. This activity is directly related to their ability to promote cell migration. A literature-based meta-analysis disclosed that SPRR proteins do not only exert this function in squamous epithelia but also in a wide range of other major tissues and organs.

## Results and Discussion

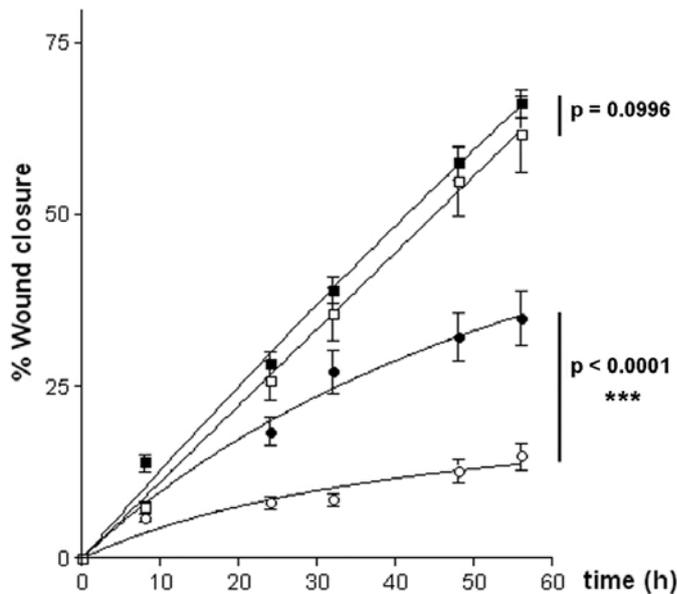
While analysing wounded three-dimensional human skin equivalents (HSE)<sup>43</sup> we found that SPRR proteins showed massive expression at the migrating front (Figure 1A; arrows), which exceeds their normal expression in the upper layers of the skin (arrowheads). SPRR proteins were preferentially found in those cells that are in close contact with the

wounded site, suggesting a role in cell migration and new tissue formation. This was substantiated by using a scratch wound assay performed on cultured human keratinocytes. Immunofluorescence staining showed, similar to the HSE data, elevated SPRR expression at the edge of the wound, localising preferentially to the cell periphery of the front row of migrating cells (Figure 1B). This pattern is observed along the whole length of the scratch, although in a patchy fashion. Indeed, the most protruding migrating cells show the highest expression of SPRR at the cell periphery (Figure 1B).



**Figure 1. Involvement of SPRR proteins in wound healing and cell migration.** A, Immunohistochemistry of SPRR1 expression (brown) in full-thickness wounds obtained after N<sub>2</sub> freezing<sup>43</sup>. Expression in the migrating front cells at the edge of the wound is indicated by large arrows. Differentiation-mediated expression in the upper layers of the epidermis is indicated by arrowheads. B, Immunofluorescence detection of SPRR1 expression (green) in cultured OKF keratinocytes 8 h after scratch-wounding. Note the preferential localisation of SPRR1 at the periphery of the cells at the edge of the scratch-wound. C, SPRR Western blot analysis of control OKF and shRNA downregulated OKF-Δ1 cells. Actin expression is used as internal control. D-G, Inhibition of cell migration in SPRR knockdown cells. OKF control (D, E) and OKF-Δ1 knockdown cells (F, G) were compared in a scratch-wound assay. Pictures D, F, were taken immediately after scratch-wounding, whereas pictures E, G, were taken after a subsequent 48 h incubation period.

This localisation at the moving edge is also supported by live-cell-imaging of pEGFP-SPRR1B transfected keratinocytes, further accentuating the presence of SPRR in membrane ruffles at the migrating front of the cell (Movie S1). In order to find evidence for a direct link between SPRR expression and cell migration, we established keratinocyte cell lines expressing shRNA specifically targeting SPRR genes (OKF- $\Delta$ 1; see Material and Methods) or carrying the empty expression vector (OKF). Under normal culture conditions both cell lines were undistinguishable and showed similar population doublings (data not shown), although in the shRNA expressing cells (OKF- $\Delta$ 1), SPRR expression was clearly down-regulated (Figure 1C). When comparing the ability of wound closure, however, a remarkable difference was perceived between the control and shRNA expressing keratinocytes. Whereas control keratinocytes started to migrate after scratch-wounding (compare Figure 1D and 1E), resulting in a substantial closure after 48 hours, this process was significantly retarded in the SPRR down-regulated cells (Figure 1F and 1G). Quantification substantiated that the shRNA expressing cells lack the ability of rapidly closing the wound (Figure 2, compare the 2 lines with open symbols), indicating that SPRR expression after wounding activates the cell migration process.



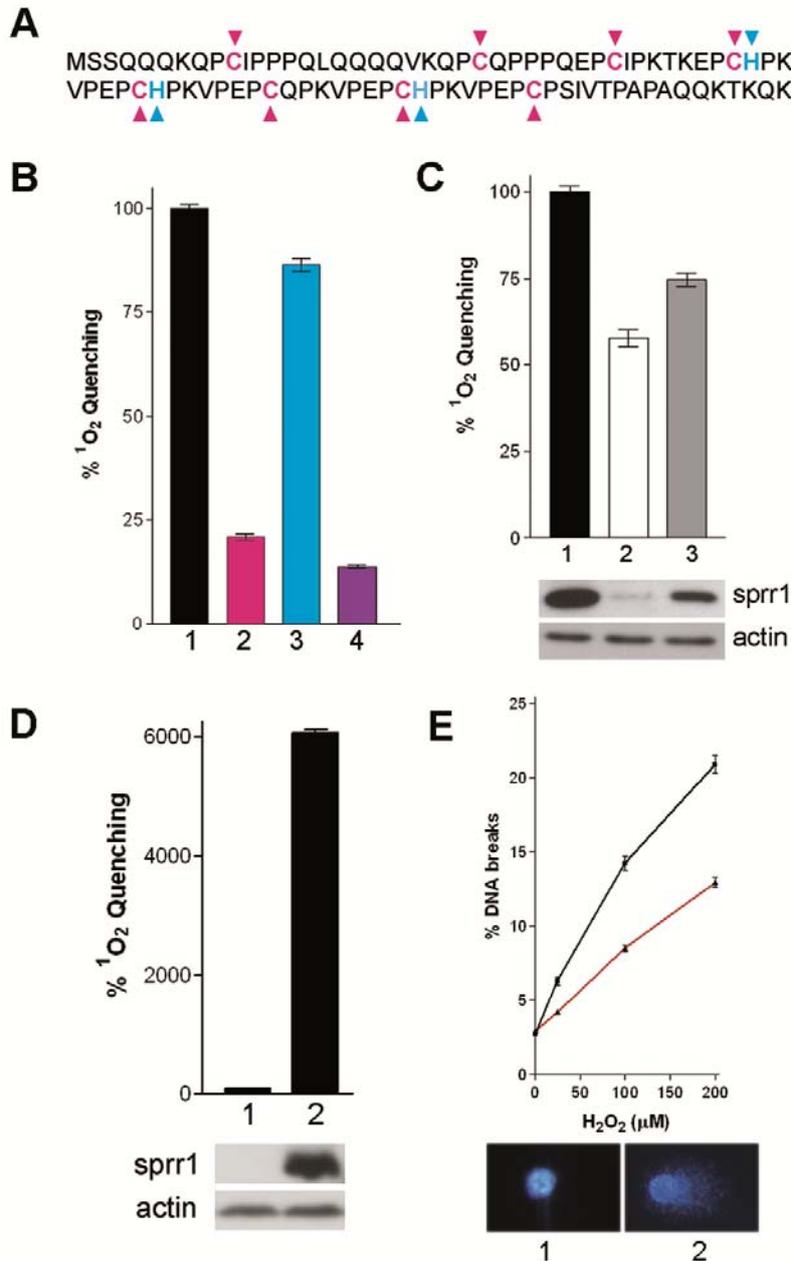
**Figure 2. Effect of ascorbate on scratch-wound closure.** Quantification of scratch closure at the indicated time-points in confluent OKF control cultures (square symbols) or OKF- $\Delta$ 1 SPRR-knockdown cells (circle symbols) either treated (solid symbols) or untreated (open symbols) with 10  $\mu$ M ascorbate following scratch-wounding. Data show mean  $\pm$  s.e.m. of at least 6 independent measurements per time-point. Curves from treated and untreated cultures were analysed via two-way ANOVA by using the Graphpad Prism software:  $p < 0.05$  values were considered to indicate a significant difference.

By taking into account the previously postulated role of SPRR proteins as ROS quenchers<sup>3</sup>, their localisation in the moving front of migrating cells directly contacting the wounded area (Figure 1) and the importance of ROS regulation and signalling during wound healing<sup>28,131,162,165</sup>, we inferred that the antioxidant properties of SPRR proteins might constitute the key for their implication in cell migration and wound healing. Consequently, it was important to first characterise the ROS quenching abilities of SPRR proteins at a molecular level. Flash-photolysis was used to quantify the life-time of singlet oxygen, a major oxidizing species in skin<sup>94</sup>, either in solution in the presence of purified SPRR proteins or directly in living cells. SPRR proteins contain besides proline high amounts of cysteine (5-18%; Figure 3A), known to be involved in ROS quenching<sup>125</sup> and signalling<sup>28,165</sup>. As expected, SPRR1B efficiently quenched singlet oxygen and this quenching was largely inhibited by specifically targeting cysteine residues with the sulfhydryl reagent N-ethylmaleimide (NEM)

(Figure 3B, compare bars 1 & 2). Histidine residues in SPRR1B contributed also to ROS quenching, but to a lesser extent (DEPC treatment, bar 3). The quenching ability of the related SPRR4 protein<sup>17</sup>, which contains cysteine but no histidine residues, was not affected by DEPC, while treatment with NEM resulted in a loss of 96% of its quenching activity (see Figure S1). These experiments directly implicate cysteine residues in ROS quenching by SPRR proteins. Flash-photolysis was also used to measure ROS quenching in living cells. OKF- $\Delta$ 1 and OKF- $\Delta$ 2 cells, where SPRR expression is downregulated to a different extent (Figure 3C), were compared to OKF control cells carrying the empty expression vector. The data clearly showed that down-regulation of SPRR results in a lower quenching potential. Similarly, ectopic expression of SPRR1B in HeLa cells, which do not express SPRR proteins, resulted in a substantial potentiating of ROS quenching (Figure 3D). The fact that these differences could be measured in the context of whole cells proves that SPRR proteins constitute a key determinant for intracellular ROS quenching. The major cellular impact of ROS in living cells is mediated via the induction of DNA strand breaks. In a comet assay<sup>26</sup> HeLa cells, ectopically expressing SPRR1B (Figure 3E, red curve), were significantly more resistant to H<sub>2</sub>O<sub>2</sub> induced DNA breaks than HeLa control cells (black curve). These experiments suggest a direct and major involvement of SPRR proteins in intracellular ROS detoxification. To assess the importance of ROS detoxification during wound healing, the scratch-wounded OKF- $\Delta$ 1 cells were treated with ascorbate, a well known ROS quencher. The loss of migratory ability due to SPRR down-regulation (Figure 2, compare open circles and squares) can at least be partially reverted by ascorbate (solid circles). Interestingly, ascorbate did not affect the migratory properties of SPRR expressing control cells (compare open and solid squares). Apparently, the enhancement of cell migration is not a general effect of ascorbate, but it is only apparent in a SPRR-deficient background, proving that SPRR mediated ROS detoxification is directly responsible for efficient cell migration.

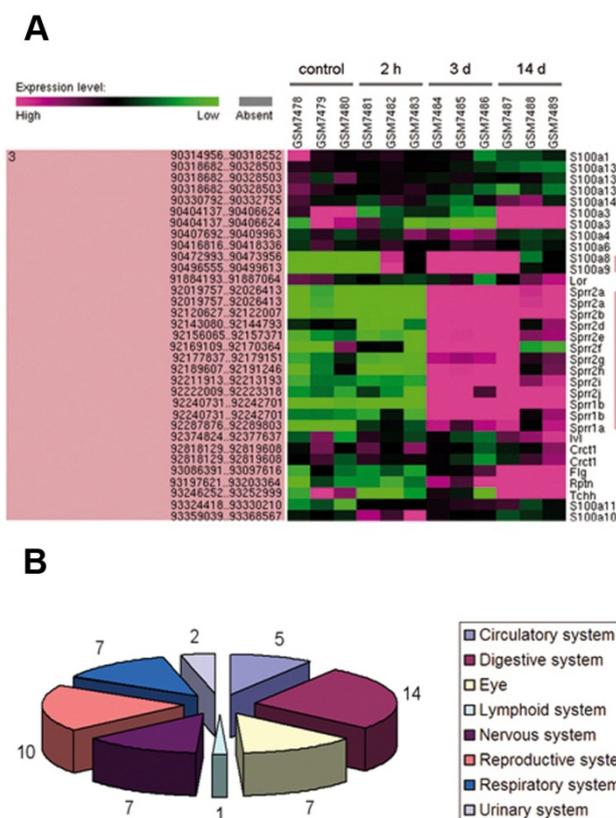
In order to further validate these *in vitro* experiments by *in vivo* observations, we performed a meta-analysis on recent genomic/proteomic screens and were able to find evidence for up-regulation of SPRR expression during wound healing in the skin. Cooper and co-workers analysed gene-expression in wounded skin from neonatal mice at different time points by clustering genes with similar expression patterns. Although not specifically mentioned by the authors, the analysis revealed several SPRRs among the most highly up-regulated genes, together with proteins with a known antioxidant function, all involved in tissue repair<sup>27</sup>. Feezor and co-workers have analyzed temporal patterns of global gene expression following infliction of murine cutaneous burn injuries<sup>47</sup>. We compared the expression of EDC genes during wound healing in their dataset available via the Gene Expression Omnibus repository (dataset GDS353; <http://www.ncbi.nlm.nih.gov/geo/>). SPRR genes (Figure 4A, large pink bar) have a very characteristic expression pattern which is clearly different from the classical cornified envelope precursor proteins involucrin and loricrin (indicated by arrows) and is only matched by S100A8 and S100A9 (short pink bar), two genes previously implicated in wound healing<sup>187</sup>.

The above data provide insight into a more global function for SPRR proteins in wound healing that might not be restricted to stratified squamous epithelia. Along these lines of thinking, we extended our literature-based meta-analysis to other tissues. This analysis



**Figure 3. Quenching of Reactive Oxygen Species by SPRR proteins *in vitro* and *in vivo*.** A, Representation in one-letter code of the human SPRR1B protein sequence: cysteine (red) and histidine (blue) residues are indicated. B, Relative singlet oxygen quenching potential of equimolar solutions of purified SPRR1B protein either untreated (bar 1, black), NEM-treated (bar 2, red), DEPC-treated (bar 3, blue) or treated with both reagents (bar 4, violet). The quenching ability of untreated protein was set at 100%. C, Quantification of singlet oxygen quenching in living OKF cells expressing various amounts of SPRR proteins. SPRR1 expression levels are indicated below the bar graph (1: control cells; 2: OKF-Δ1; 3: OKF-Δ2). D, Quantification of singlet oxygen quenching in living HeLa cells. Both control (bar 1) and HeLa cells overexpressing SPRR1B<sup>69</sup> (bar 2) are shown. E, Measurement of DNA strand breaks induced in HeLa control cells (black graph) and cells ectopically expressing SPRR1B (red graph) by using the comet assay<sup>26</sup>. For each cell line at least 200 individual cells were quantified. Inserts 1 and 2 represent respectively untreated and H<sub>2</sub>O<sub>2</sub> treated single HeLa cells stained with DAPI fluorescent DNA stain.

proved that SPRR genes are highly up-regulated in more than 50 microarray screens from divergent tissues and cell types (Figure 4B), often linked to stress or tissue remodelling. For example, in cardiomyocytes, SPRR was identified as a cardioprotective protein after ischemic stress<sup>140</sup>. During ischemia/reperfusion high levels of ROS are produced in the heart, which can be detoxified by massively increased SPRR expression in the same way as shown here for cultured cells. SPRR protein levels increase by more than 200 fold in response to bacterial colonization of the intestine<sup>73</sup> and here SPRRs are also likely to fulfil an antioxidant function. Bile duct ligation resulted in SPRR expression and subsequent adaptation of the biliary barrier<sup>133</sup>. It is interesting to mention that also in this specific case a direct link has been laid between SPRR expression and *in vitro* cell migration of human biliary epithelial cells<sup>34</sup>. During development of the mammary gland<sup>128</sup> as well as during nerve regeneration after axotomy<sup>13</sup>, SPRR proteins appear to function as tissue remodellers. In the injured axons, SPRR expression was mainly found in the axonal growth cones where it localises to cellular membrane ruffles, very similar to the situation described here for migrating keratinocytes. Additionally the same study has revealed that down-regulation of SPRR in axotomized neurons impaired directed axonal outgrowth<sup>13</sup>.



**Figure 4. Meta-analysis of SPRR expression during cutaneous wound healing and in non-squamous tissues.** A, Effect of cutaneous burn wounding on gene expression in the epidermal differentiation complex (EDC) on mouse chromosome 3. Microarray data are from the Gene Expression Omnibus repository (<http://www.ncbi.nlm.nih.gov/geo/dataset/GDS353>). Expression levels of 4 experimental conditions are shown: unburned skin (control), burn skin harvested 2 hours (2h), 3 days (3d) and 14 days (14d) after injury<sup>47</sup>. The positions of involucrin and loricrin are indicated by arrows. The SPRR gene family and the S100A8 and S100A9 genes are marked with pink bars. B, Graphical representation of the number of studies describing SPRR up-regulation in non-squamous tissues (grouped per organ) following stress or injury. The corresponding references are supplied in Table 2, Chapter I.

The above data disclose a novel and unexpected role of SPRR proteins in global wound healing, which links ROS protection and tissue remodelling. How can this function be reconciled with their canonical function restricted to skin keratinisation? Previously we have proposed that the differential regulation of the 11 members of the SPRR gene-family

provides a molecular mechanism for effectively adapting the barrier function in the uppermost layer of our skin. This differential regulation of highly homologous genes with redundant functions that respond selectively to various forms of stress or tissue requirements, allows a tightly regulated protein dosage that provides optimal barrier function to different squamous epithelia, while allowing adaptation to diverse external insults<sup>18</sup>. In the same way, a similar protein dosage mechanism is also likely to provide all tissues with an efficient, finely tuneable antioxidant barrier, specifically adapted to the tissue involved and the damage inflicted. The recognition of SPRRs as efficient cell protective proteins, linking ROS detoxification with cell migration, will provide new possibilities to study and manage tissue repair and wound healing at a molecular level.

## Materials and Methods

### Human skin equivalents

Sections from full-thickness human skin equivalent wounds, induced by liquid nitrogen freezing, were obtained from Dr. A. El Ghalbzouri (Department of Dermatology, Leiden University Medical Centre) and were previously described<sup>43</sup>. Expression of SPRR was assessed by immunostaining using a monospecific rabbit-antibody<sup>69</sup> and counterstained with hematoxylin. The mono-specificity of the antibodies was tested on HeLa cells (which do not express SPRR proteins), transfected with the various SPRR family members, both for Western blotting and immunostaining<sup>69</sup>.

### shRNA mediated knockdown

For the downregulation of SPRR, we constructed an episomal expression vector, named pESuper, derived from pECV25 (ATCC) and a fragment of pSuper<sup>15</sup> containing the H1-RNA promoter and polylinker sequence. Oligonucleotides containing the SPRR specific target sequences GCAGTGCAAGCAGCCCTGC (siRNA1) and CGCCAAAGTGCCAGAGCC (siRNA2) were used for the shRNA-mediated knockdowns. They target both SPRR1 and SPRR2 genes. Immortal OKF keratinocytes (OKF6/TERT-2<sup>35</sup>), which do not express SPRR3 and SPRR4 (our unpublished observations) were kindly provided by Dr. J.G. Rheinwald (Harvard Medical School, Boston), cultured in defined Keratinocyte-SFM (KSFM; GIBCO) as described<sup>35</sup> and transfected with the above mentioned pESuper constructs, or empty vector control, by using the Amaxa nucleofector according to the manufacturer's protocol (Lonza AG, Cologne, Germany). Stable cell-lines, designated OKF-Δ1 (OKF cells containing the pESuper vector with siRNA1), OKF-Δ2 (containing pESuper with siRNA2), and OKF control cells (empty pESuper vector), were grown until confluence. One day thereafter scratch wounds were made with a yellow pipette tip, the medium was exchanged for KSFM without growth additives and photographs were taken at the indicated time-points. The area

remaining free of cells was quantified using Adobe Photoshop and Biorad Quantity One software.

### Protein chemistry

SPRR proteins were produced by using IPTG induction of *Escherichia coli* BL21 (DE3)\*RP (Stratagene, La Jolla) bacteria transformed with a pET-vector (Novagen) containing a full-length SPRR cDNA insert. Bacterial pellets were lysed by freeze-thawing in 25mM sodium-citrate (pH 3.6), 1mM EDTA, 1mM DTT. Under these conditions SPRR proteins remained soluble. Upon centrifugation at 37,000 rpm in a Ti60 rotor (Beckman) the supernatant was further purified using a 6 ml Resource S column (GE Healthcare). The buffer was exchanged to 10mM sodium-phosphate (pH 7.0) and the SPRR proteins were stored at -80°C. Sulfhydryl groups of cysteines were modified with N-ethylmaleimide (NEM) and histidine residues with diethylpyrocarbonate (DEPC). The purity of all proteins as well as all modifications were confirmed by mass spectrometry.

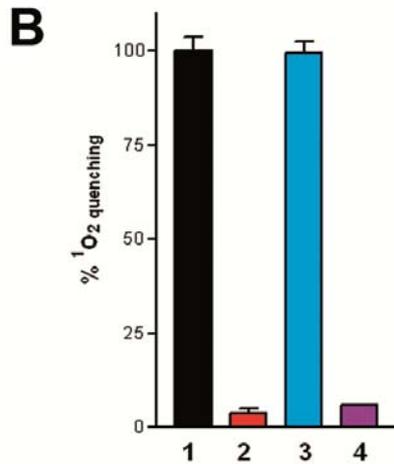
### ROS measurements and toxicity assays

Flash-photolysis experiments were performed using a Continuum Surelite I YAG-laser.  $^1\text{O}_2$  was detected with a Judson Germanium G-050 photodiode coupled to a Judson preamplifier. All samples were measured in a glass cuvette with magnetic stirrer in  $\text{D}_2\text{O}$  containing Rose Bengal as sensitizer. The comet assay was performed as previously described<sup>26</sup> and quantified using ColourProc, an in-house software program kindly provided by Dr. H. Vrolijk (Department of Molecular Cell Biology, Leiden University Medical Center).

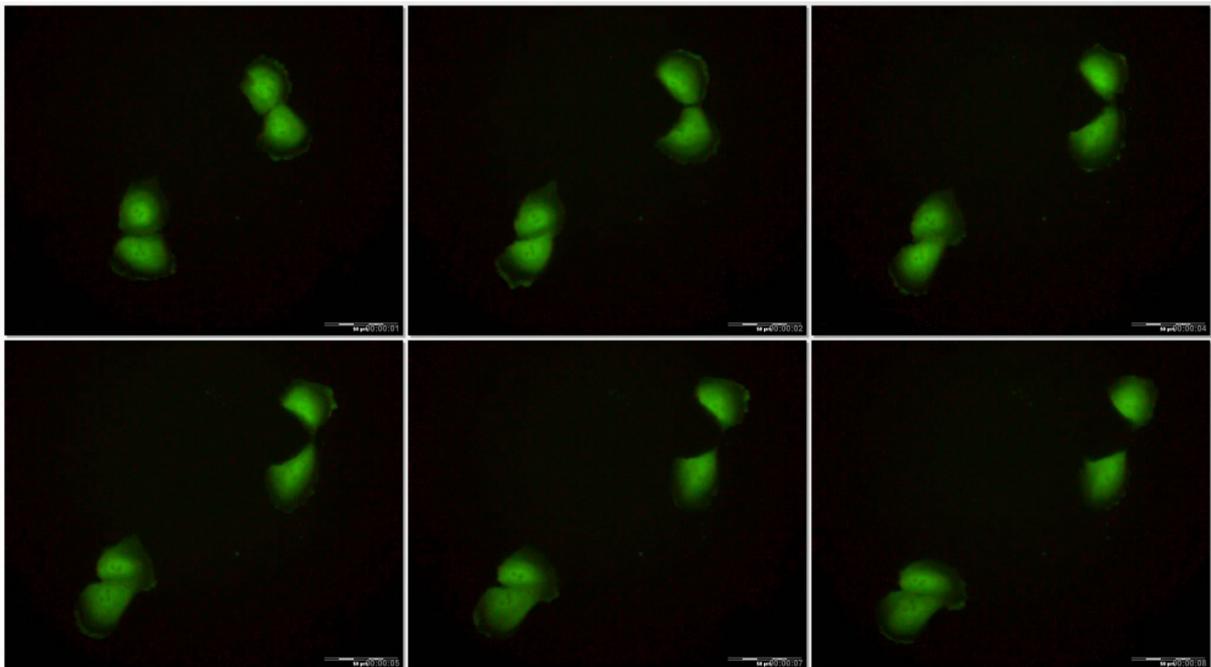
### Acknowledgements

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Supporting information is available at PLoS ONE online.  
<http://www.plosone.org/>



**Figure S1. Reactive oxygen quenching of SPRR4 protein in vitro.** A, Representation in one-letter code of the human SPRR4 protein sequence: cysteine residues are indicated in red. Note that SPRR4 does not contain histidine residues. B, Relative singlet oxygen quenching potential of equimolar solutions of purified SPRR4 protein either untreated (bar 1, black), NEM-treated (bar 2, red), DEPC-treated (bar 3, blue) or treated with both reagents (bar 4, violet). The quenching ability of untreated protein was set at 100%.



**Movie S1. Involvement of SPRR proteins in cell migration.** OKF keratinocytes were transiently transfected with pEGFP-SPRR1B and analyzed with the Olympus IX81 live-imaging station. Time-lapse images were recorded for 200 min at intervals of 2 min. Note the localization of SPRR proteins to membrane ruffles at the leading edge of migrating cells. This pattern was not observed in parallel cultures expressing pEGFP-actin or pEGFP-tubulin fusion proteins and was confirmed by SPRR-antibody staining. SPRR proteins were also consistently found in the nuclei of these cells.



# Chapter IV

*ROS quenching potential of the epidermal  
cornified cell envelope.*

Wilbert P. Vermeij, A. Alia and Claude Backendorf  
*J. Invest. Dermatol.* 2011; 131 (7): 1435-1441

### Abstract

The cornified cell envelope (CE) is a specialised structure assembled beneath the plasma membrane of keratinocytes in the outermost layers of the epidermis. It is essential for the physical and permeability properties of the skin's barrier function. Our skin is continuously exposed to atmospheric oxygen and threatened by reactive oxygen species (ROS). Here we identify the CE as a first line of antioxidant defence and show that the small-proline-rich (SPRR) family of CE precursor proteins play a major role in ROS detoxification. Cysteine residues within these proteins are responsible for ROS quenching, resulting in inter- and intramolecular S-S bond formation, both in isolated proteins and purified CEs. The related keratinocyte-proline-rich protein (KPRP) is also oxidized on several cysteine residues within the CE. Differences in antioxidant potential between various SPRR family members are likely determined by structural differences rather than by the amount of cysteine residues per protein. Loricrin, a major component of the CE with a higher cysteine content than SPRRs, is a weak ROS quencher and oxidized on a single cysteine residue within the CE. It is inferred that especially SPRR proteins provide the outermost layer of our skin with a highly adaptive and protective antioxidant shield.

## Introduction

Reactive oxygen species (ROS) can have beneficial effects as they operate as regulatory molecules in multiple intracellular signalling pathways, for instance as the first danger signal during wound healing to attract immune cells, or merely as chemical sterilizers in our host defence mechanism<sup>28,48,118</sup>. Nevertheless in general ROS are considered as toxic compounds. In the mid-1950s, Denham Harman proposed ROS as essential determinants of the ageing process, since excessive ROS can damage lipids, proteins and nucleic acids leading to cellular dysfunction and death<sup>65</sup>. More recently, altered ROS levels were implicated in diseases such as Alzheimer, Parkinson, atherosclerosis, rheumatoid arthritis, diabetes, psoriasis, cystic fibrosis, hypertension, ischemia and cancer<sup>12,202</sup>.

Of all tissues, our skin is exposed to the highest ROS levels. It is, besides the lungs and eyes, the only organ in direct contact with atmospheric oxygen, including air pollutants and the natural deleterious ozone gas. The skin has been shown, as early as 1851, to directly take-up oxygen via cutaneous respiration<sup>194</sup>. Skin also faces high levels of ROS which are induced during wound healing against invading bacteria<sup>118</sup>. In addition, various types of ROS, like the superoxide anion ( $O_2^{\cdot-}$ ), the hydroxyl radical ( $HO\cdot$ ), hydrogen peroxide ( $H_2O_2$ ) and singlet oxygen ( $^1O_2$ ) are generated following exposure to UV radiation derived from natural sunlight<sup>139,164</sup>. In order to cope with excessive ROS and to endow a protective antioxidant barrier, our skin has evolved several detoxification mechanisms<sup>95,167</sup>. These antioxidants can be classified into two major groups, enzymes and low molecular weight antioxidants (LMWA). The LMWA group contains compounds such as ascorbic acid (vitamin C), tocopherol (vitamin E), uric acid, glutathione, and ubiquinol, all capable of directly scavenging ROS<sup>95</sup>. The enzyme group contains superoxide dismutase, catalase, peroxidase and glutathione reductase, which in turn can actively detoxify ROS<sup>122,164</sup>. Beside the two major antioxidant classes, other proteins function in this detoxification process, either by direct quenching or indirect regulation of signalling pathways that activate the antioxidant defence system<sup>28,202</sup>.

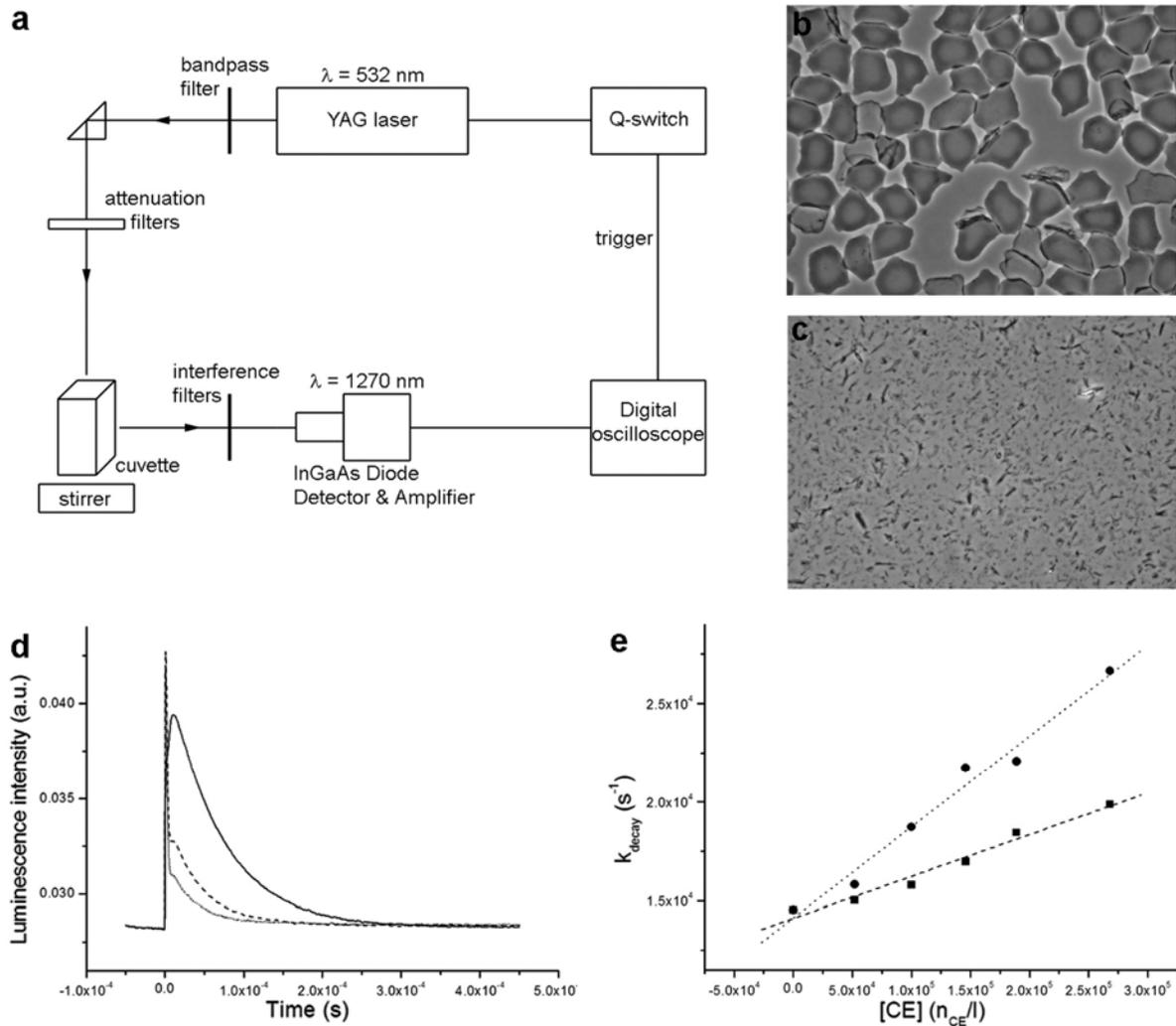
Many antioxidants are present at higher levels in the epidermis as compared to the dermis, correlating with decreasing ROS levels towards the inner layers of our skin<sup>167</sup>. In fact it was shown that the most external, cornified layer of the skin already provides sufficient antioxidant protection following a challenge with for instance ozone<sup>186</sup>. Within the cornified layer, a specialised structure surrounding the terminally differentiated keratinocytes, named the cornified cell envelope (CE), is responsible for the physical and permeability properties of the skin's innate barrier function<sup>19,84</sup>. During the epidermal differentiation process several proteins are expressed from the epidermal differentiation complex (EDC) localised on human chromosome 1q21 (e.g. involucrin, loricrin and the SPRR or LCE protein families)<sup>18,19</sup>. These cornified envelope precursor proteins contain highly similar head and tail domains, rich in lysine and glutamine, which are involved in transglutaminase-mediated cross-linking in the outermost layers of the skin<sup>10,84</sup>. On cross-linking on the cell periphery they form, together with lipids, the CE<sup>130,147</sup>.

We recently showed that SPRR proteins are able to detoxify ROS during wound healing<sup>192</sup>. Interestingly, this novel function of SPRR is not only restricted to squamous epithelia. Indeed, on injury, SPRR protein expression massively increases at the edge of the wound in various types of tissues. This increase directly lowers the amount of ROS at the wounded site and is essential to allow proper cell migration during the wound healing process. Since SPRR proteins evolved together with all other EDC genes for their role in the assembly of the CE<sup>190</sup>, we inferred that the antioxidant potential of the SPRR proteins could also provide the skin with an antioxidant barrier. In this paper, we identify the CE as a first line of antioxidant defence, as it is able to directly quench ROS. We show that the SPRR family of CE precursor proteins, which were originally identified as UV-inducible genes<sup>86</sup>, play a major role in ROS quenching both *in vitro* and *in vivo*, mainly due to their cysteine residues.

## Results and Discussion

In a first instance we examined the potency of purified CEs in ROS quenching. CEs from sunburned peeled skin were isolated as previously described<sup>123</sup> and measured with an in-house Flash-photolysis set-up, which is graphically represented in Figure 1a. With this technique it is possible to quantitatively measure the time-resolved near-infrared luminescence of singlet oxygen, one of the major oxidizing species in skin<sup>94</sup>. All reactions were performed in a glass cuvette with magnetic stirrer, containing Rose Bengal (RB), to generate singlet oxygen, and D<sub>2</sub>O, allowing a longer singlet oxygen lifetime<sup>89</sup>. The advantage of this system is that the quenching potential of any compound, whether it is a purified protein, a living cell, or in this case, isolated intact (Figure 1b) or sonicated CEs (Figure 1c) can be quantified. A typical time-profile of the singlet oxygen luminescence in D<sub>2</sub>O is shown in Figure 1d (solid line). Addition of intact (dashed line) or sonicated CEs (dotted line) both resulted in a substantial decrease in the singlet oxygen lifetime, indicating their direct involvement in ROS quenching. The rate constant of singlet oxygen decay, which can be calculated from the slopes in Figure 1e, is significantly increased in sonicated CEs (dotted line) as compared to intact ones (dashed line). The CE consists of a protein envelope coated by a lipid envelope<sup>68,130</sup>. Sonication is likely to result in a better accessibility of the internal proteinaceous CE components, suggesting that the protein part of the CE might be responsible for the antioxidant properties of the CE. It can, however, not be completely excluded that oxidation products, possibly generated during sonication, also contribute to the higher quenching ability of sonicated CEs. Analysis of CEs from different body sites has previously revealed that lorocrin and the SPRR protein family together always comprise about 85-90 percent of the total CE protein mass with relative molar ratios ranging from >100-1 in trunk epidermis to 5-1 in footpad epidermis and 3-1 in forestomach epithelium<sup>93,174</sup>.

In order to compare the individual antioxidant properties of these major CE precursor components *in vivo*, stable cell lines were established expressing lorocrin (HFLor), SPRR1B



**Figure 1. Flash-photolysis detection of the singlet oxygen quenching by cornified cell envelopes.** **a**, Graphical representation of the used Flash-photolysis set-up. Singlet oxygen is produced by laser excitation at 532nm of the samples, containing  $\text{D}_2\text{O}$  and Rose Bengal as sensitizer. The subsequent decay of singlet oxygen is measured at 1270nm with a photodiode. **b-c**, Photographs of purified, intact (**b**) and sonicated (**c**) cornified cell envelopes isolated from human skin. Scale bar = 15  $\mu\text{m}$ . **d**, Typical time-profile of the luminescence of singlet oxygen (solid line), which is significantly reduced after addition of intact (dashed line) or sonicated CEs (dotted line). **e**, Singlet oxygen decay ( $k_{\text{decay}}$ ) plotted against increasing concentrations of intact (dashed line) or sonicated CEs (dotted line).

(HF1B), SPRR2A (HF2A), SPRR3 (HF3), SPRR4 (HF4) or empty vector control (H24) in HeLa cells, which do not express these proteins (our unpublished observation). It appeared that each of these ectopically expressed proteins potentiated the ROS quenching ability of the transfected cells (Table 1). The relative percentage of quenching was calculated by dividing the cellular quenching rate constants by the respective protein expression levels determined via Western blotting. The highest effect was observed with cells expressing SPRR4 (set at 100%), followed by the other SPRR proteins (approximately 50% efficiency) and lorycin with a relative effect of 25%. To verify the differences in ROS quenching and relate them directly

to the various proteins in question, the Flash-photolysis measurements were repeated with purified proteins. Due to the insolubility of loricrin it was not possible to purify this protein and include it in the *in vitro* study. As far as SPRRs are concerned, similar rate constants were identified for SPRR1B, SPRR2A, and SPRR3 (Table 2), and again a higher value was observed for SPRR4, consistent with the cellular data. This indicates that the data obtained in living cells (Table 1) are the result of direct ROS quenching by the ectopically expressed proteins.

**Table 1.** *In vivo* quenching of reactive oxygen species by living cells ectopically expressing loricrin or one of the SPRR proteins

Cell line	Rate constant <sup>1</sup>	SD	Relative expression <sup>2</sup>	Relative % quenching <sup>3</sup>
HFLor	7,540E-02	6,03E-03	1,76	25,3
HF1B	4,169E-01	1,02E-02	4,71	52,3
HF2A	2,274E-01	2,49E-02	2,86	47,0
HF3	1,898E-01	1,14E-02	2,18	51,4
HF4	1,692E-01	1,22E-02	1,00	100,0
H24	6,900E-03	7,72E-04	- <sup>4</sup>	-

<sup>1</sup> *In vivo* rate constants ( $L \times C^{-1} \times s^{-1}$ ) obtained by Flash-photolysis with cultured cells are depicted in singlet oxygen lifetime (L) per cell (C) per second (s).

<sup>2</sup> The relative expression levels were calculated by western blot. The band intensities were quantified after detection with a FLAG antibody and the expression level of FLAG-SPRR4 was set at 1.

<sup>3</sup> The relative % quenching was calculated by correcting the individual cellular rate constants for the expression level of the particular proteins

<sup>4</sup> – The value could not be calculated since no protein was ectopically expressed in this cell line.

**Table 2.** *In vitro* quenching of reactive oxygen species by purified SPRR proteins

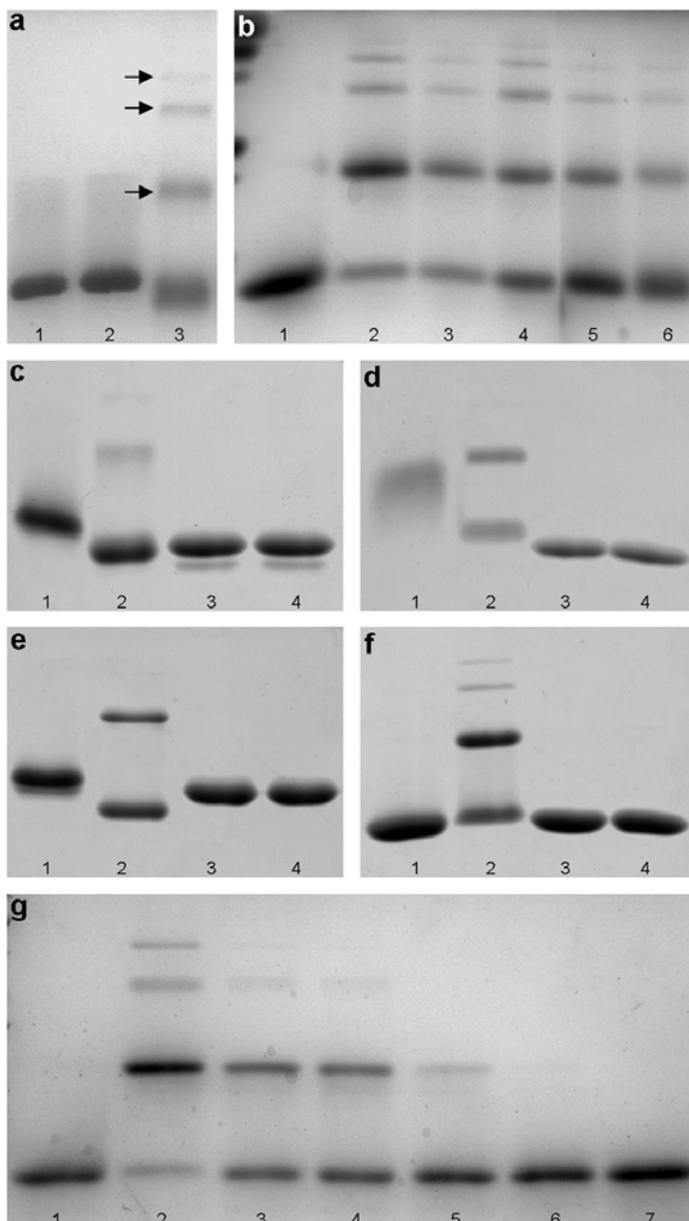
Protein	Rate constant <sup>1</sup>	SD	Relative % quenching	No. of cysteines
Loricrin	ND	ND	ND	19
SPRR1B	6,078E+08	9,75E+07	50,9	8
SPRR2A	6,755E+08	4,62E+07	56,5	11
SPRR3	6,318E+08	1,11E+08	52,9	8
SPRR4	1,195E+09	1,63E+08	100	7
SPRR4NEM	4,650E+07	3,46E+06	3,9	- <sup>2</sup>

<sup>1</sup> *In vitro* rate constants ( $L \times M^{-1} \times s^{-1}$ ) obtained by Flash-photolysis with purified proteins are depicted in singlet oxygen lifetime (L) per mole protein (M) per second (s).

<sup>2</sup> All cysteine residues in this sample were inactivated by NEM modification. The extent of modification was confirmed by mass spectrometry.

While performing these *in vitro* experiments we observed that singlet oxygen, produced via illumination of RB, induced SPRR protein multimerisation (Figure 2a, lane 3 arrows). This multimerisation was not observed in the presence of RB without irradiation (lane 2), indicating that the production of ROS directly affects the SPRR proteins. Indeed, various other oxidising compounds induced the formation of similar SPRR multimers (Figure

2b). All SPRR proteins contain besides proline residues, high amounts of cysteine, a known redox-regulated amino acid involved in ROS quenching and signalling in many proteins<sup>28,125</sup>. Specific inactivation of the cysteine residues in SPRR1B (Figure 2c), SPRR2A (Figure 2d), SPRR3 (Figure 2e) and SPRR4 (Figure 2f) by N-ethylmaleimide (NEM) prevented both inter- and intramolecular S-S bond formation (compare lanes 2 and 4). Addition of ROS to the untreated SPRR proteins (lanes 1) resulted in the formation of dimers and trimers (lanes 2), and tetramers in the case of SPRR4. Interestingly, SPRR1B, SPRR2A, and SPRR3 appear also to be subjected to intramolecular S-S bond formation, as shown by the appearance of protein forms migrating faster than the monomeric form. SPRR multimerisation is gradually reverted upon addition of increasing amounts of  $\beta$ -mercaptoethanol (Figure 2g). To prove the direct implication of cysteine residues and S-S bond formation in ROS quenching, the singlet oxygen decay rate of NEM treated SPRR4 was measured. The results revealed that by specifically inactivating all cysteine residues in SPRR4 the quenching activity was almost completely inhibited (Table 2; bottom row).



**Figure 2. SPRR multimerisation induced by cysteine oxidation.** **a**, PAGE analysis of untreated (lane 1), Rose Bengal treated (lanes 2, 3) and illuminated SPRR4 (lane 3). Arrows indicate dimer, trimer, and tetramer formation. **b**, Multimerisation of SPRR4 by various types of ROS: no treatment (lane 1), hydrogen peroxide (lane 2), peroxyradical (lane 3), bromate radical (lane 4) illuminated Toluidine Blue (lane 5), Chlorin e6 (lane 6). **c-f**, H<sub>2</sub>O<sub>2</sub> induced multimerisation of SPRR1B (**c**), SPRR2A (**d**), SPRR3 (**e**), and SPRR4 (**f**) is inhibited by N-ethylmaleimide: mock-treated (lane 1), H<sub>2</sub>O<sub>2</sub> (lane 2), NEM (lane 3); NEM followed by H<sub>2</sub>O<sub>2</sub> (lane 4). **g**, Reversion of H<sub>2</sub>O<sub>2</sub>-mediated SPRR4 multimerisation (lane 2) with increasing concentrations of  $\beta$ -mercaptoethanol (3-7). Lane 1: untreated control.



To ascertain whether S-S bond formation is actually responsible for ROS quenching in isolated CEs (shown in Figure 1), the oxidation state of cysteine residues in CE components was analysed by LTQ-Orbitrap tandem mass spectrometry. Sonicated CEs were first mock or H<sub>2</sub>O<sub>2</sub> treated and subsequently modified with NEM, which only reacts with the free thiol groups. The remaining oxidised cysteines were reduced by addition of dithiothreitol and then modified with iodoacetamide, a second thiol reactive compound. In this way cysteine residues engaged in S-S bonds within the CE are selectively modified with iodoacetamide whereas free thiol groups are modified with NEM. Following tryptic digestion, many peptides containing oxidation sensitive cysteine residues were found. By only gating iodoacetamide labelled peptides originating from EDC genes or known cornified envelope precursor genes (Table 3), multiple cysteine-containing SPRR peptides appeared to be subjected to H<sub>2</sub>O<sub>2</sub> induced oxidation, demonstrating that SPRR proteins fulfil a major role in ROS detoxification within the CE. In addition, cysteine-containing peptides originating from loricrin and filaggrin-2, one of the fused gene family members in the EDC<sup>142</sup>, were identified to be involved in the formation of S-S bonds. One of the most recently identified EDC genes, keratinocyte proline-rich protein (KPRP)<sup>97</sup> showed, similar to SPRR, numerous oxidised cysteines residues. To our knowledge, this is the first direct evidence that KPRP is a CE precursor protein and that it is involved in ROS detoxification.

**Table 3.** Mass spectrometric identification of CE peptides involved in S-S bonds after oxidation

Sequence	$\Delta$ p.p.m.	Peptide score	z	Protein
CPEPC*PPPK	-0,87	49	2	SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR2G
C*PEPCPPPK	0,91	41	2	SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR2G
C*PPVTPSPPC*QPK	1,48	60	2	SPRR2E, SPRR2F
QPC*QPPVC*PTPK	0,41	46	3	SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2G
QPTPQPPVDC*VK	-1,25	51	2	Loricrin
FGGQGNQFSYIQSGC*QSGIK	-0,19	94	2	Filaggrin-2
GGQGHGC*VSGGQPSGC*GQPESN PC*SQSYSQR	1,85	127	3	Filaggrin-2
C*PVEIPPIR	1,12	60	2	Keratinocyte proline-rich protein
IEISSPC*C*PR	-1,81	59	2	Keratinocyte proline-rich protein
GRPAVC*QPQGR	0,35	36	3	Keratinocyte proline-rich protein
LDQC*PESPLQR	-1,47	66	2	Keratinocyte proline-rich protein
FSTQC*QYQGSYSSC*GPOFQSR	-0,23	60	3	Keratinocyte proline-rich protein
TSFSPC*VPQC*QTQGSYGSFTEQHR	0,3	80	3	Keratinocyte proline-rich protein
LDTEAPYC*GPSSYNQGQESGAGC* GPGDVFPER	-0,29	117	3	Keratinocyte proline-rich protein

Cysteine residues involved in the formation of S-S bonds were identified via iodoacetamide labelling (see Materials and Methods) and are indicated by C\*. Mass deviations between measured and theoretical values of the various peptides are given as  $\Delta$ p.p.m. (absolute values <5 are considered as accurate measurements). The respective peptide scores (reflecting the reliability of the assignment of the peptide sequence) and z-values (charge of the peptide) are also provided. All Mascot identifications were manually validated and gated against a subset of the SwissProt database containing EDC and known CE precursor proteins (a list of these proteins is provided as supplementary information).

Overall, our data indicate that all SPRR proteins have the ability to contribute to the antioxidant properties of the CE, but to a different extent, depending on the amount and accessibility of their cysteine residues. Our data suggest that the CE, as part of the outermost layer of our skin, constitutes the first line of antioxidant defence and provides protection against the high levels of ROS engendered by atmospheric oxygen and UV irradiation. UV light for instance can induce ROS production and trigger lipid peroxidation<sup>46,132</sup>. Within the stratum corneum the lipid envelope is in close contact with the proteinaceous CE components<sup>130</sup>. In this way the majority of ROS can be directly secured by the SPRR CE precursor proteins at the periphery of cells. This will permit a more efficient handling of residual cell-infiltrating ROS by the LMWA and enzymatic antioxidants. Besides triggering ROS production, UV-irradiation of the skin has previously been shown to induce SPRR4 expression associated with thickening of the stratum corneum<sup>17</sup>. As the SPRR proteins can be differentially regulated by a complex panel of interdependent transcription factor complexes<sup>50</sup>, this stress-induced activation is likely part of the skin's antioxidant defence against subsequent ROS damage. We have previously inferred that the differential regulation of highly homologous SPRR proteins constitutes the basis for an adaptive epithelial barrier function. The data provided here demonstrate that SPRR proteins also provide the outermost layer of our skin with a highly adaptive and protective antioxidant shield.

## Materials and Methods

### Flash-photolysis

Singlet oxygen,  $O_2(^1\Delta_g)$ , quenching rate constants ( $k_q$ ) were determined by monitoring its time-resolved luminescence following Nd:YAG laser (Continuum, Santa Clara) excitation of Rose Bengal at 532 nm. This generates singlet oxygen via energy transfer from the triplet state of Rose Bengal to ground state molecular oxygen. The subsequent luminescence at 1270 nm was detected with a Judson Germanium G-050 photodiode coupled to a Judson preamplifier (Judson Technologies, Montgomeryville, PA). All samples were measured in a glass cuvette with magnetic stirrer in 300 $\mu$ l  $D_2O$  (Merck, Darmstadt, Germany) with 5 $\mu$ l 10mM Rose Bengal (Sigma-Aldrich, St Louis, MO) added before any quencher was supplied. The luminescence decay of singlet oxygen was averaged over 256 measurements per concentration of quencher and was independently measured for at least six different concentrations per sample. At each concentration a luminescence trace was obtained and fitted with a single exponential. The quenching rate constants for purified CEs, SPRR proteins and CE precursor expressing cells were calculated from the singlet oxygen decay rates ( $k_{decay}$ ) plotted against the concentrations of quencher. The cellular rate constants were divided by the respective protein expression levels, quantified by Western-blot with a monoclonal anti-FLAG antibody (clone M5, Sigma), to obtain the relative percentage of quenching.

### **SPRR protein production and purification**

SPRR proteins were produced by using isopropyl- $\beta$ -D-thio-galactoside induction of *E. coli* BL21 (DE3)\*RP (Stratagene, La Jolla) bacteria transformed with a pET-vector (Merck) containing a full-length SPRR cDNA sequence. Bacterial pellets were lysed by freeze-thawing in 25mM sodium-citrate (pH 3.6), 1mM EDTA, 1mM dithiothreitol in which the SPRR proteins remained soluble. Upon centrifugation at 37,000 rpm in a Ti60 rotor (Beckman-Coulter, Brea, CA) the supernatant was further purified using a 6ml Resource S column (GE Healthcare, Diegem, Belgium). The buffer was exchanged to 10mM sodium-phosphate (pH 7.0) and the SPRR proteins were stored at -80°C. The purity of all proteins was confirmed by mass spectrometry.

### **SPRR multimerisation and cysteine modification**

All multimerisation experiments were performed using identical molar ratios of proteins and oxidising compounds. Singlet oxygen was generated by illumination of Rose Bengal (RB), Toluidine Blue (TB) or Chlorine e6 (Ce6), for two minutes with a 500 watt halogen lamp. Treatments with all other oxidising compounds were for a period of 10 minutes. The following final concentrations were used: 10mM H<sub>2</sub>O<sub>2</sub>; 0.2mM FeSO<sub>4</sub> with 1mM H<sub>2</sub>O<sub>2</sub>; 10mM KBrO<sub>3</sub>; 10mM RB; 0.5mM TB; 10mM Ce6. All reactions were performed on ice, and equal amounts of protein were loaded on gel, using loading buffer without  $\beta$ -mercaptoethanol. Cysteine residues of SPRR were specifically inactivated by incubation with 20mM N-ethylmaleimide (NEM; Sigma-Aldrich) for one hour. The modifications were confirmed by mass spectrometry.

### **Secondary structure predictions**

Secondary structure predictions were performed with the BetaTPred2 web server<sup>87</sup> (<http://www.imtech.res.in/raghava/betatpred2/>).

### **Cell culture**

For the generation of stable cell lines full-length SPRR or loricrin cDNA sequences were provided with an N-terminal FLAG-tag and introduced into the episomal expression vector pECV25<sup>11</sup>. HeLa cells, which do not express any of these proteins, were transfected using DOTAP (Boehringer, Mannheim, Germany), and were grown in DMEM supplemented with 10% newborn bovine serum, Pen/Strep, and 300  $\mu$ g/ml hygromycin. Stable cell lines were named HFLor (loricrin), HF1B (SPRR1B), HF2A (SPRR2A), HF3 (SPRR3), HF4 (SPRR4) and H24 (empty vector control). For intracellular protein quantification the various ectopically expressed proteins were detected on Western blots with monoclonal anti-FLAG antibody (clone M5, Sigma).

## CE isolation and mass spectrometric characterisation

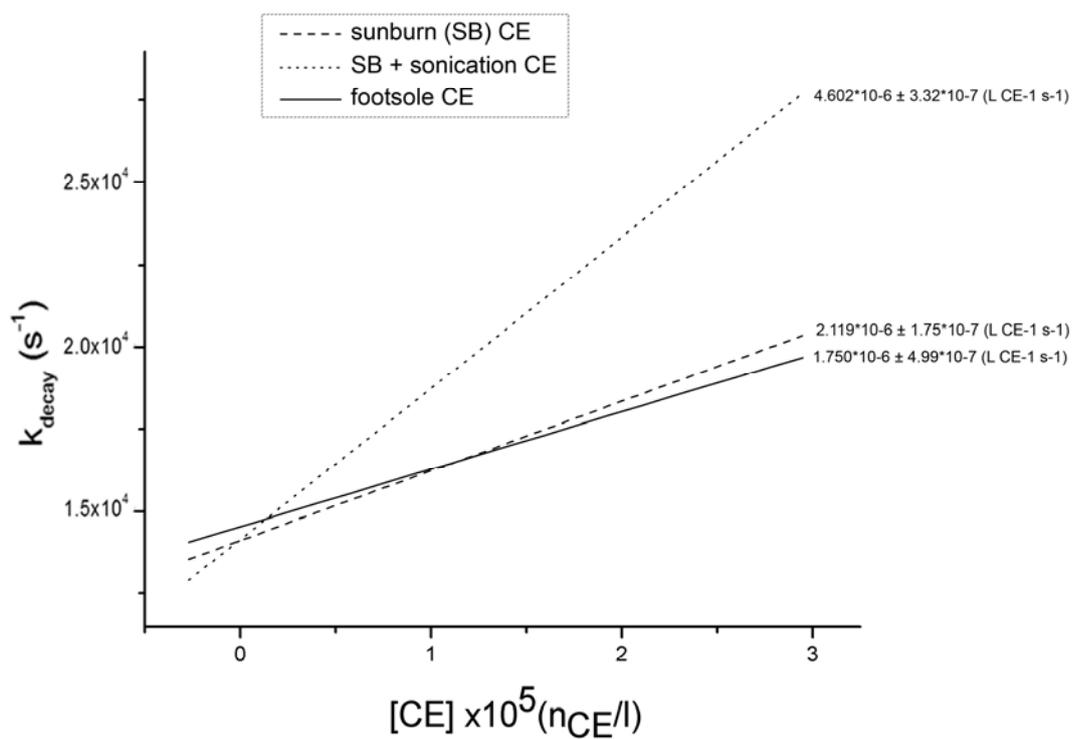
Cornified cell envelopes were isolated from sun-burned peeled skin according to the previously described procedure<sup>123</sup> by boiling the skin pieces in 100mM Tris-Cl (pH 7.5), 2% SDS, 1mM EDTA, 10mM DTT. Note the reducing character of this buffer (10 mM DTT), which means that CEs will lose all oxidised cysteine residues during the purification procedure and are as such isolated in their native form (as composites of transglutaminase-crosslinked proteins and lipids).

For mass spectrometry determination sonicated CEs were oxidized with 10mM H<sub>2</sub>O<sub>2</sub> followed by NEM.treatment. In this way, all cysteine residues that are in a position to form S-S bonds will become oxidised, whereas all non-oxidized cysteines will be modified with NEM. The samples were subsequently reduced with dithiothreitol and treated with iodoacetamide to modify the originally oxidised cysteines. The subsequent procedures and treatments for tryptic digestion, stage-tip purification, LTQ-Orbitrap tandem mass spectrometry and Mascot analysis are described elsewhere<sup>53</sup>. Within Mascot all identified peptides were characterised via the SwissProt database. All protein identifications were manually validated and gated for EDC and known CE precursor proteins. A list of these reference proteins is provided as supplementary information.

## Acknowledgements

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Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>



**Figure S1:** Singlet oxygen decay ( $k_{\text{decay}}$ ) plotted against increasing concentrations of intact (dashed line) or sonicated CEs (dotted line) isolated from sunburned peeled skin or from footsole (solid line). The respective rate constants are indicated beside the various lines.



# Chapter V

*Proteomic identification of in vivo interactors reveals novel functions of skin cornification proteins.*

Wilbert P. Vermeij, Bogdan I. Florea, Sheena Isenia, A. Alia, Jaap Brouwer and Claude Backendorf  
*Manuscript submitted*

### Abstract

Protection against injurious external insults and loss of vital fluids is essential for life and is in all organisms, from bacteria to plants and humans, provided by some form of barrier. Members of the small-proline-rich (SPRR) protein family are major components of the cornified cell envelope (CE), a structure responsible for the barrier properties of our skin. These proteins are efficient reactive oxygen species (ROS) quenchers involved not only in the establishment of the skin's barrier function but also in cell migration and wound healing. Here, a proteomic analysis of *in-vivo* SPRR-interacting proteins confirmed their function in CE-formation and ROS-quenching and also revealed a novel unexpected role in DNA-binding. Direct *in-vitro* and *in-vivo* evidence proved that the DNA-binding capacity of SPRRs is regulated by the oxidation state of the proteins. At low ROS levels, nuclear SPRR is able to bind DNA and prevent ROS-induced DNA damage. When ROS levels increase SPRR proteins multimerize and form an effective antioxidant barrier at the cell periphery, possibly to prevent the production or infiltration of ROS. At even higher ROS exposure DNA-binding is restituted. A molecular model explaining how the intracellular oxidation state of SPRRs likely influences their selective protective function is provided.

## Introduction

The small proline-rich (SPRR) gene family consists of 11 highly homologous members which are clustered within the epidermal differentiation complex (EDC) localized on human chromosome 1q21<sup>18</sup>. Together with other EDC genes (e.g. loricrin, involucrin and the LCE gene family), the SPRRs are expressed in the upper layers of cornifying tissues<sup>18,19</sup>. All of these genes share significant sequence similarity as they contain lysine- and glutamine-rich N- and C-terminal domains which are utilized for transglutaminase cross-linking during the building-up of the cornified cell envelope (CE)<sup>10,84</sup>. This insoluble structure, formed at the inside of terminally differentiated keratinocytes, is responsible for the major barrier properties of the skin<sup>19,147</sup>. It provides protection against biological, chemical and mechanical insults, prevents the loss of vital fluids and is essential for mammalian life<sup>19</sup>.

As the SPRR family members can be differentially regulated by a complex panel of transcription factors, SPRR protein dosage can be rapidly modulated upon a variety of physiological and environmental stimuli<sup>50</sup>. By varying the amount of incorporated SPRR protein, the biomechanical properties of the CE can be regulated<sup>18,174</sup>. Increased SPRR protein levels within the CE generally results in a more strengthened barrier which provides enhanced resistance to external stressors.

In addition to this canonical function in the establishment of the epidermal barrier, we recently showed that SPRR proteins are expressed throughout our body in a variety of non-cornifying epithelia<sup>192</sup>. Within these tissues, SPRR proteins are not cross-linked by transglutaminases in a fixed CE-like structure, but form a reversible barrier via disulfide bond formation<sup>191,192</sup>. Upon tissue damage by destructive stimuli, reactive oxygen species (ROS) are generated as a defense against invading bacteria and as signaling molecules initiating the healing process<sup>9,64</sup>. As a result, the amount of SPRR proteins massively increases, which in turn directly detoxifies ROS and promotes cell migration. While quenching ROS via their cysteine residues, SPRR proteins multimerize and form an antioxidant shield at the cell periphery that protects cellular components and the tissue as a whole<sup>192</sup>. Likewise, SPRR proteins confer antioxidant properties to the CE and form the skin's first line of antioxidant defense against atmospheric oxygen and UV irradiation<sup>191</sup>.

We have previously shown that SPRR proteins are efficient ROS quenchers and provide protection against ROS induced DNA damage<sup>191,192</sup>. In this paper, we have examined the molecular mechanism behind this process.

## Materials and Methods

### Protein interaction screens

For the isolation and identification of protein interaction complexes, SPRR1B and SPRR4 were provided with a Strep-tag and used as bait proteins. Full-length SPRR cDNA sequences were cloned in the pEXPR-IBA105-vector as described by the manufacturer (IBA, Göttingen, Germany). Immortal OKF keratinocytes (OKF6/TERT-2) were kindly provided by Dr. J.G. Rheinwald (Harvard Medical School, Boston) and cultured in defined Keratinocyte-SFM (KSFM; GIBCO) as described by Dickson et al.<sup>35</sup>. Transfections with the above mentioned pEXPR-constructs, or empty vector controls, were performed by using Amaxa nucleofection according to the manufacturer (Lonza AG, Cologne, Germany). Approximately  $10^9$  cells were lysed in cold 50mM Tris-HCl (pH 7.5), 5mM EDTA, 250mM NaCl, 0.1% Triton X-100, 7mM  $\text{CaCl}_2$  supplemented with 5mM NaF, 100mM  $\text{Na}_3\text{VO}_4$ , 20mM  $\beta$ -glycerolphosphate, and 1 protease inhibitor cocktail tablet (Roche) per 10ml buffer, all freshly added before use. The soluble cell extracts were loaded on a 0.2ml Strep-Tactin column (IBA, Göttingen, Germany) and the unbound proteins were washed away. Subsequently, a first elution step was performed with 0.5ml wash-buffer (100mM Tris-HCl (pH 8.0), 150mM NaCl, 1mM EDTA) supplemented with 50mM DTT. In this way, proteins interacting via or stabilized by disulfide bonds were eluted. Residual interaction partners or complexes were eluted together with the Strep-tagged SPRR bait protein by using biotin elution buffer: 100mM Tris-HCl (pH 8.0), 150mM NaCl, 1mM EDTA, 2mM biotin.

### Mass spectrometry analysis and database searching

Proteins from the individual elution fractions were precipitated by addition of equal volumes of 20% trichloroacetic acid<sup>121</sup>. After three wash-steps with 0.2ml ice cold acetone, the proteins were dissolved in 25 $\mu$ l 8M Urea, 0.4M ammonium bicarbonate. The Cysteine residues were reduced with 5 $\mu$ l 45mM DTT for 15 min at 50°C and alkylated with 5 $\mu$ l 100mM iodoacetamide at room temperature in the dark. Trypsin digestion was performed at 37°C overnight<sup>121</sup>, and the resultant peptides were purified using StageTips<sup>145</sup>. The LTQ-Orbitrap (Thermo Fisher Scientific, Waltham, MA) tandem mass spectrometry analysis was performed as previously described<sup>53</sup>.

Database searching with Mascot (Matrix Science, Boston, MA) against all human entries in Swiss-Prot was performed with the following parameters: peptide tolerance 2ppm, MS/MS tolerance 0.5Da, fixed carbamidomethyl modification (C), variable oxidation (M), 2 missed cleavages allowed, decoy database option on. The MudPIT scoring algorithm was used with an ion score cutoff of 20, with required bold red only. Two unique peptide assignments with a significance threshold value  $p < 0.05$  were required per protein identification. The datasets were imported in Pipeline Pilot (Accelrys Inc., San Diego, CA) in which the respective DTT and biotin elution fractions were combined, the empty vector control dataset was subtracted, and a protein cutoff score of 40 was applied. The resulting lists of potential binding partners (Table S1) were explored with the WEB-based Gene-SeT-AnaLysis Toolkit (WebGestalt) (<http://bioinfo.vanderbilt.edu/webgestalt/index.php>) and the enriched Gene Ontology categories were identified by the statistics module<sup>208</sup>.

### SPRR4 cellular localization and toxicity assays

HeLa cells ectopically expressing FLAG-tagged SPRR4 (HF4), SPRR1B (HF1b), or empty vector control (H24) were cultured as previously described<sup>191</sup>. The localization of SPRR4 was assessed by immunostaining using a monospecific rabbit-antibody, obtained after immunization of rabbits with the peptides DPCAPQVKKQCPPKG and CPSAQQASKSKQK (Eurogentec). The mono-specificity of these antibodies was assessed as previously described<sup>69</sup>. Hoechst 33342 (Sigma) was used as DNA stain. HF4-cells were pre-treated with 50 $\mu$ M H<sub>2</sub>O<sub>2</sub> for the indicated time points and fixed with cold 80% acetone.

Comet assays were performed as previously described<sup>26,192</sup> and quantified using ColourProc, an in-house software program kindly provided by Dr. H. Vrolijk (Department of Molecular Cell Biology, Leiden University Medical Center, Leiden). The H<sub>2</sub>O<sub>2</sub> concentrations used ranged from 0 to 200 $\mu$ M with at least 1000 comets measured per cell-line. From the linear increase in DNA breaks, the calculated slopes were used as a value for the Intracellular Quenching Activity (IQA) of the specific cell-lines. For intracellular protein quantification, the various ectopically expressed proteins were detected on Western blot with a monoclonal anti-FLAG antibody (clone M5, Sigma).

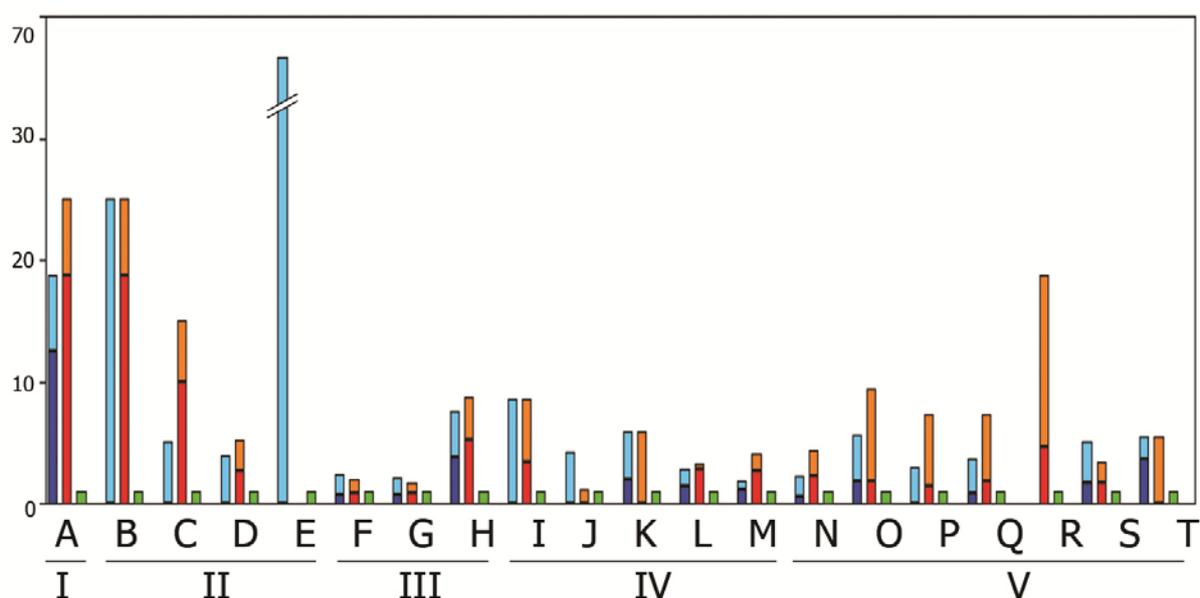
### SPRR protein multimerization and DNA binding assays

SPRR proteins were produced and purified as previously described<sup>192</sup>. For all DNA binding experiments 0.12 pmoles of a linear DNA fragment (3200 bp) was used with a 100 times molar excess of SPRR4 protein (12 pmoles). Prior to DNA binding, equal amounts of protein stock solutions were oxidized using a serial dilution of H<sub>2</sub>O<sub>2</sub> ranging from 0 to 100mM. Excess H<sub>2</sub>O<sub>2</sub> was removed by gel filtration on Sephadex G10 spin columns or diluted out in binding buffer. All binding reactions were performed on ice in 10mM sodium phosphate buffer (pH7) and analyzed on 1% agarose gels (for DNA detection) or 15% PAGE (for protein detection), using loading buffer without  $\beta$ -mercaptoethanol. Binding of SPRR4 to DNA was stable until a salt concentration of 100mM NaCl (results not shown). For the analysis of the redox state of the cysteine residues within these diversely oxidized SPRR samples, the different protein bands were cut into small pieces. The free thiol groups were first labeled with N-ethylmaleimide as previously described<sup>191</sup>. Subsequently, the cysteines engaged in disulfide bonds were reduced by addition of DTT and labeled with iodoacetamide. In this way free thiols can be recognized by N-ethylmaleimide labeling and cysteines originally engaged in disulfide bonds by iodoacetamide. Thiols already oxidized to sulfenic-, sulfinic- or sulfonic acid are not affected by these treatments. The labeled SPRR4 peptides were extracted after in-gel tryptic digestion<sup>166</sup> and analyzed by OT-Orbitrap tandem mass spectrometry<sup>53,191</sup>. Cysteine modifications of SPRR4 peptides were manually identified in Xcalibur (Thermo Fisher Scientific, Waltham, MA) by using the SPRR4 protein sequence (NCBI accession no. AF335109) and the calculated peptide masses. All identified peptides were validated by de novo sequencing using PEAKS (Bioinformatics Solutions Inc., Waterloo, ON, Canada).

## Results

## Identification of SPRR interacting proteins

SPRR proteins were subjected to a protein-protein interaction screen. Strep-tagged SPRR proteins, used as bait, were expressed in human OKF keratinocytes, an immortalized cell line normally expressing these proteins upon epidermal differentiation or cell migration<sup>192</sup>. Soluble cell extracts were loaded on a Strep-Tactin column and after removal of all non-interacting proteins, SPRR interactors were isolated using a dual elution procedure to distinguish between different modes of interaction. In step 1 wash buffer supplemented with DTT was used to identify proteins whose interaction is mediated or stabilized via disulfide bonds. In step 2 biotin elution buffer was used to elute the remaining interacting proteins. Eluted samples were analyzed by LTQ-Orbitrap tandem mass spectrometry and the potential binding partners were identified using the Mascot search engine<sup>53,137</sup>. Peptide hits from the empty vector control were subtracted and the identified interactors (Table S1) were characterized by the WebGestalt program<sup>208</sup>.



**Figure 1.** Graphical representation of the Gene Ontology molecular function annotations of the identified SPRR interacting proteins. The proteins isolated using SPRR1B (light/dark blue bars) or SPRR4 (orange/red bars) as bait were characterized by their Gene Ontology molecular function annotations. The ratio of enrichment was calculated by dividing the observed gene number for the specific SPRR protein by the expected gene number (green bars; set at one) for each category. The light blue and orange part of the bars represents the fraction isolated using wash-buffer supplemented with DTT, whereas the dark blue and red part represents the fraction isolated with biotin elution buffer. The GO terms for each category which were at least two-fold enriched are given in Table 1.

Among the gene ontology (GO) molecular function terms, twenty categories (A-T) were enriched by more than two-fold for at least one of the SPRRs (Fig. 1). In fact, almost all GO categories were enriched for both SPRR1B (light/dark blue bars) and SPRR4 (orange/red bars), as compared to the expected number of genes calculated by the program (green bars; set at one). These categories (Table 1) encompass molecular function terms such as "structural constituent of epidermis" (A), "peroxiredoxin activity" (B), "protein disulfide isomerase activity" (C), "structural constituent of cytoskeleton" (H), "intramolecular transferase activity" (I), and "double-stranded DNA binding" (O). Interestingly, the categories "thioredoxin peroxidase activity" (E) and "DNA bending activity" (R), which clearly belonged to the highest scores, were selectively enriched for either SPRR1B or SPRR4 respectively. Several GO annotations were closely related or represented already known functions of the SPRR protein family and were therefore clustered (clusters I-III and V in Figure 1 and Table 1).

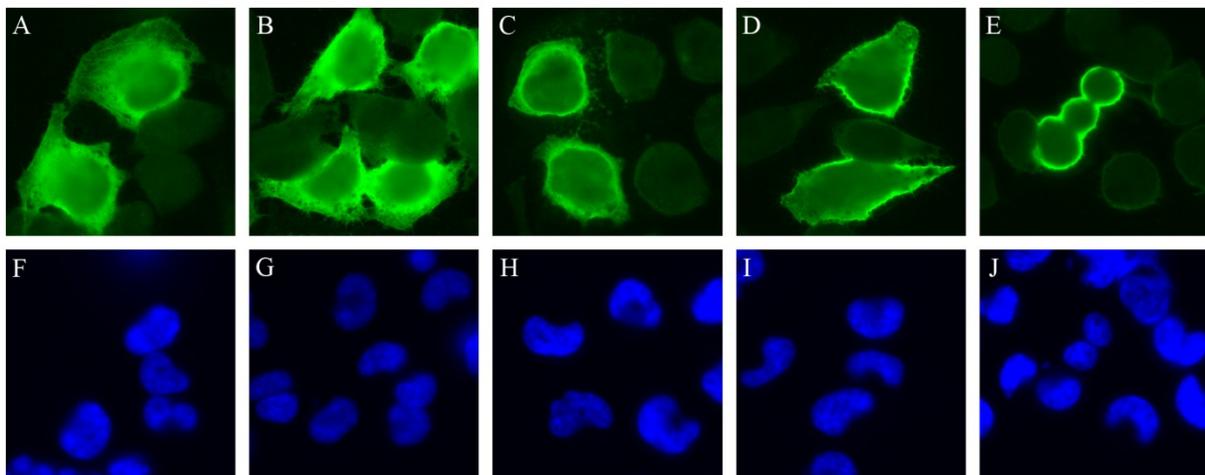
**Table 1.** Individual and clustered Gene Ontology categories describing the molecular function terms of Figure 1.

Letter	GO annotation	Cluster
A	Structural constituent of epidermis	I
B	Peroxiredoxin activity	II
C	Protein disulfide isomerase activity	II
D	Protein disulfide oxidoreductase activity	II
E	Thioredoxin peroxidase activity	II
F	Actin binding	III
G	Cytoskeletal protein binding	III
H	Structural constituent of cytoskeleton	III
I	Intramolecular transferase activity	IV
J	Protein phosphatase regulator activity	IV
K	Calcium-dependent phospholipid binding	IV
L	Unfolded protein binding	IV
M	Translation regulator activity	IV
N	Nucleic acid binding	V
O	Double-stranded DNA binding	V
P	Single-stranded DNA binding	V
Q	Structure-specific DNA binding	V
R	DNA bending activity	V
S	DNA helicase activity	V
T	RNA helicase activity	V
Cluster	Molecular function term	
I	Epidermal cornification	
II	ROS quenching	
III	Cytoskeletal protein binding	
IV	Diverse	
V	Nucleic acid binding	

The remaining set of GO terms, which could not be grouped under a single denominator, contains diverse broad molecular functions (cluster IV). The identified groups contain one molecular function term involved in epidermal cornification, four terms in ROS quenching, three in cytoskeletal protein binding, and seven in nucleic acid binding (Table 1). Especially this latter molecular function was unexpected.

### Cellular localization of SPRR is subjected to ROS treatment

To ascertain whether SPRR proteins are actually involved in the identified molecular functions, their cellular localization was analyzed. Immunofluorescence staining with a SPRR4 monospecific antibody revealed mainly cytoplasmic localization in HeLa cells ectopically expressing FLAG-tagged SPRR4 (HF4) (Fig. 2). Within the cytoplasm, ordered fiber-like structures can be observed (Fig. 2A), indicating that SPRR proteins are at least in close proximity to some cytoskeletal proteins. In addition, minor but consistent nuclear SPRR4 staining was observed (Fig. 2A-C). Immunofluorescence staining against the N-terminal FLAG-tag showed similar cytoplasmic and nuclear localization, further proving the specificity of the SPRR4 antibody (data not shown). Interestingly, the nuclear localization of SPRR4 changed upon  $H_2O_2$  treatment of the cells (Fig. 2A-E). After a ROS challenge, SPRR proteins shifted towards the cytoplasm where they preferentially localized to the cytoplasmic membrane. Mock treatment followed by a similar incubation period did not result in this altered localization (data not shown).



**Figure 2.** The cellular distribution of SPRR4 is affected by ROS. Immunofluorescence detection of SPRR4 expression (A-E) in HeLa cells ectopically expressing FLAG-tagged SPRR4 (HF4). DNA was counterstained with Hoechst (F-J). The cells were subjected to ROS treatment with  $50\mu M H_2O_2$  for a time period of 0 (A,F), 5 (B,G), 10 (C,H), 15 (D,I), or 20 (E,J) minutes.

We have previously shown that SPRR1B protects against  $H_2O_2$  induced DNA breaks<sup>192</sup>. Hence we investigated also the effect of SPRR4 on ROS induced DNA breakage and compared it to SPRR1B. As a control, the increase in DNA breaks after addition of various

concentrations of H<sub>2</sub>O<sub>2</sub> to empty vector control cells (named H24) was analyzed using a comet assay. From the linear increase in DNA breaks after addition of H<sub>2</sub>O<sub>2</sub>, the calculated slope was used as a value for the intracellular quenching activity (IQA) of a specific cell-line (Table 2). Ectopic expression of SPRR1B (HF1b) resulted in a decrease in the amount of DNA breaks and consequently in a lower slope and thus a higher IQA. Although the expression level of SPRR4 (HF4) was clearly lower as compared to SPRR1B, it gave a similar reduction in the amount of DNA breaks, indicating its superior relative intracellular quenching activity (RIQA) (almost 5-fold increase; Table 2, last column).

**Table 2.** Quantification of ROS induced DNA breaks in HeLa cells ectopically expressing SPRR1B, SPRR4 or empty vector control cells.

Ectopic protein	Cell line	Slope ( $\Delta$ DNA Breaks/[H <sub>2</sub> O <sub>2</sub> ])	IQA <sup>1</sup>	Relative expr. level	RIQA <sup>2</sup>
none	H24	0,089 ± 0,009	11,15	-	1,00
SPRR1B	HF1b	0,050 ± 0,002	19,84	1	1,78
SPRR4	HF4	0,049 ± 0,001	20,08	0,21	8,58

<sup>1</sup> Intracellular Quenching Activity (IQA) is calculated as 1/slope

<sup>2</sup> Relative IQA (RIQA) is corrected for the relative expression level of the ectopically expressed SPRR proteins

At least 1000 comets were analyzed for each cell line.

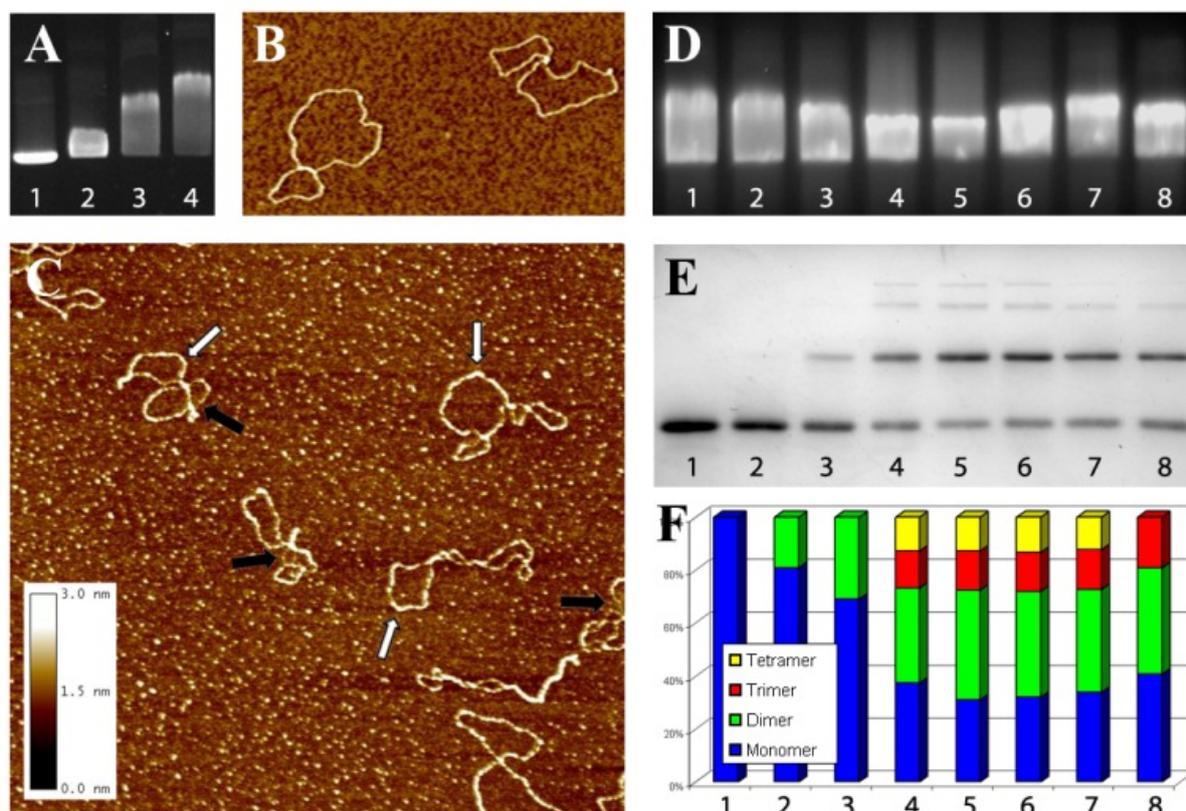
### SPRR proteins bind directly to DNA

In order to evaluate a possible direct binding of SPRR proteins to DNA, electrophoretic mobility shift assays were performed with purified SPRR proteins and isolated DNA fragments. Addition of increasing amounts of purified SPRR4 protein to a linear DNA fragment resulted in a lower mobility of the DNA molecules in agarose gel (Fig. 3A). Similar electrophoretic mobility shifts were observed when using purified SPRR1, SPRR2, or SPRR3 proteins, although a 10 times higher molar excess of protein was required (data not shown). These data imply that SPRR proteins have the ability to directly bind to double-stranded DNA. This ability was further substantiated by using atomic force microscopy (AFM). Circular DNA was visualized in the absence (Fig. 3B) and presence (Fig. 3C) of SPRR4. The analysis indicates that SPRR4 has the ability to randomly coat the DNA double helix (coated – and uncoated DNA regions are indicated by respectively white and black arrows). Both experiments constitute the first direct evidence that SPRR proteins have the ability to bind directly to DNA. They further corroborate the protein interactome analysis and the cellular localization studies described above.

### The oxidation state of SPRR proteins influences their DNA binding capacity

To investigate the effect of ROS on the DNA binding properties of SPRR proteins, equal amounts of purified SPRR4 were pre-treated with various concentrations of H<sub>2</sub>O<sub>2</sub>. At

initial increasing concentrations of  $H_2O_2$  a clear gradual decrease in DNA binding was observed (Fig. 3D, lanes 1-5). Analysis of the corresponding fractions via PAGE revealed an increase in the formation of SPRR multimers (Fig. 3E, lanes 1-5), illustrated by a decrease of SPRR monomers and an increase of dimers, trimers and tetramers (Fig. 3F, bars 1-5). However, when higher  $H_2O_2$  concentrations were used (lanes 6-7) a reversion of the above mentioned effect was observed as the DNA binding capacity of SPRR4 increased again (Fig. 3D, lanes 6-7), while the amount of SPRR multimers decreased (Fig. 3E/F, lanes 6-7). Surprisingly, the highest  $H_2O_2$  concentration showed a second drop in DNA binding (Fig. 3D, lane 8), whereas the amount of multimers was still decreasing (Fig. 3F, bar 8).



**Figure 3.** DNA binding properties of SPRR proteins depend on their oxidation state. **A**, addition of increasing amounts of purified SPRR4 protein to 0.12 pmoles of a linear DNA fragment (3200 bp) results in an altered migration in agarose gel. Zero, 50, 100, and 150 times molar excess of SPRR4 proteins were respectively used in lanes 1 to 4. **B-C**, analysis of the SPRR4-DNA complexes with AFM. Circular DNA in the absence (**B**) and presence of purified SPRR4 (**C**). Black and white arrows respectively indicate native DNA regions or DNA regions decorated by SPRR proteins. **D-F**, SPRR protein multimerization and DNA binding are affected by ROS. DNA binding of SPRR4 proteins, oxidized with increasing concentrations of  $H_2O_2$  (1: 0mM, 2: 2.4mM, 3: 4.7mM, 4: 9.0mM, 5: 17.5mM, 6: 33mM, 7: 64mM, 8: 124mM), is shown in panel **D**; the same SPRR fractions were also analysed on PAGE (panel **E**). The relative amounts of monomers (blue), dimers (green), trimers (red) and tetramers (yellow) on the protein gel of panel **E** are quantified in panel **F**.

Disulfide bonds can be broken either by reduction to free thiols or by further oxidation to sulfenic-, sulfinic-, and sulfonic acids<sup>138,202</sup>. These latter forms are characterized by different electronic properties which can result in altered protein activities<sup>138</sup>. To examine the effect of these different adducts on SPRR multimerization and DNA binding the redox state of the individual protein forms was analyzed. The cysteine residues within the different protein bands of lanes 1, 5, and 8 (Fig. 3E) were subjected to a dual labeling step with two different thiol-specific compounds to distinguish the various oxidation states (see Experimental Procedures). Following tryptic digestion, the extracted SPRR peptides were analyzed by OT-Orbitrap tandem mass spectrometry and the redox state of the cysteine residues was determined. The results of table 3 indicate that all 7 cysteine residues within SPRR4 have the ability to form disulfide bonds following ROS treatment and that the majority could be further oxidized to sulfinic-, and/or sulfonic acids. The generally unstable sulfenic acid intermediate was not detected.

**Table 3.** Identified oxidation states of the 7 cysteine residues in SPRR4.

Sequence	Observed oxidation states	$\Delta$ p.p.m.	Reduced <sup>1</sup>	Oxidized <sup>2</sup>	Highly oxidized <sup>3</sup>
QQQQC <sub>13</sub> PPQR	Thiol	-0,15	Dark	Dark	Dark
	Disulfide	0,48	Empty	Empty	Empty
	Sulfonic acid	0,16	Empty	Empty	Empty
QPC <sub>27</sub> QPPPVK	Thiol	0,66	Dark	Dark	Empty
	Disulfide	-0,72	Empty	Empty	Dark
	Sulfinic acid	-2,36	Empty	Empty	Dark
	Sulfonic acid	0,95	Empty	Empty	Dark
C <sub>34</sub> QETC <sub>38</sub> APK	Thiol/Thiol	0,74	Dark	Dark	Empty
	Thiol/Disulfide	-0,62	Empty	Empty	Dark
	Disulfide/Disulfide	-0,05	Empty	Empty	Dark
DPC <sub>46</sub> APQVK	Thiol	0,65	Dark	Dark	Empty
	Disulfide	-0,73	Empty	Empty	Dark
	Sulfinic acid	-0,55	Empty	Empty	Dark
	Sulfonic acid	1,45	Empty	Empty	Dark
QC <sub>54</sub> PPK	Thiol	-0,01	Dark	Empty	Empty
	Disulfide	-0,97	Empty	Dark	Dark
	Sulfinic acid	0,19	Empty	Empty	Dark
C <sub>67</sub> PSAQQASK	Thiol	-0,52	Dark	Dark	Empty
	Disulfide	0,11	Empty	Dark	Empty

The various SPRR4 tryptic peptides containing cysteine residues (indicated in red) are represented. The number following C represents the position in the primary amino acid sequence (NCBI accession no. AF335109). The various protein oxidation states are derived from the protein samples loaded on gel in Figure 3E and treated with the following H<sub>2</sub>O<sub>2</sub> concentrations: <sup>1</sup> lane 1 (none); <sup>2</sup> lane 5 (17.5 mM); and <sup>3</sup> lane 8 (124 mM). Mass deviations between measured and theoretical values of the various peptides are given as  $\Delta$ p.p.m. (absolute values < 5 are considered as accurate measurements). Dark fields: detected; empty fields: not detected.

## Discussion

### Interaction of SPRR with proteins involved in epidermal cornification and ROS quenching

As building blocks of the CE, SPRR proteins are cross-linked to other CE precursor proteins thereby providing our skin with a highly adaptive and protective barrier function<sup>18,19</sup>. In order to be able to detect all possible SPRR interacting proteins in our interactome screen we have used an immortalized keratinocyte cell line which is still able to differentiate<sup>35</sup> and express SPRR and other CE precursor proteins. We have previously shown that these cells do also express SPRR proteins during cell migration in a scratch-wound assay<sup>192</sup>. In our screen several of the identified proteins such as desmoplakin, desmoglein, and S100 calcium binding proteins are known structural constituents of the epidermis (Fig. 1, bars A). Interacting proteins were isolated using a dual elution procedure which distinguishes between different modes of interaction. The first fraction, eluted with DTT, represents proteins whose interaction is mediated or stabilized solely via disulfide bonds. Most structural CE proteins were eluted in the second fraction using biotin elution buffer. These proteins are indeed known to be cross-linked in the CE by transglutaminases via their lysine and glutamine residues<sup>19,111</sup>, which explains their appearance in our protein interaction screen.

Beside their role in epidermal cornification, SPRR proteins also fulfill an important role in the detoxification of ROS, both in the upper layers of the skin and throughout the whole body during tissue remodeling<sup>191,192</sup>. This is accentuated by the identification of numerous proteins with peroxiredoxin activity, protein disulfide isomerase or oxidoreductase activity, or thioredoxin peroxidase activity (Fig. 1, cluster II). All these GO terms mainly originated from the first elution fraction (disulfide group), in line with the ROS quenching of SPRR proteins via their cysteine residues<sup>192</sup>. It is interesting to mention that in clusters I and II most GO terms were upregulated both for SPRR1B and SPRR4 with the only exception of the thioredoxin activity (bar E) which was associated solely with SPRR1B and not with SPRR4. Thioredoxin has the ability to reduce inter- and intra-molecular S-S bonds<sup>28</sup> and might as such promote SPRR1B protein turnover. Nevertheless, our previous analysis has indicated that SPRR4 is the better ROS quencher<sup>191</sup>. Inter- and intramolecular disulfide bonds have also been detected in native CEs isolated from human skin<sup>191</sup> proving that our *in vitro* screening procedure revealed physiologically relevant interactions. These interactions are reversible *in vitro* following  $\beta$ -mercaptoethanol treatment<sup>191</sup>.

### SPRR proteins function in DNA binding

Besides the GO categories described above, other less expected molecular function terms appeared in our screen. Cluster III contains cytoskeletal binding proteins which appear to interact with both SPRR1B and SPRR4. These interactions are further substantiated by the fact that SPRR4 localizes to ordered fiber-like structures within the cytoplasm of OKF keratinocytes (Fig. 2A). Apparently, SPRR proteins are at least in close proximity to some cytoskeletal proteins. Both Pradervand et al.<sup>140</sup> and Bonilla et al.<sup>13</sup> have previously described

the localization of SPRR1 along actin structures. Although no direct physical interaction was detected, it was inferred that SPRR proteins might alter cytoskeletal functions and contribute to tissue remodeling<sup>13,140</sup>. Consistent with this, we recently showed that SPRR proteins indeed localize to membrane ruffles of migrating keratinocytes and play a major role in cell migration during wound healing<sup>192</sup>.

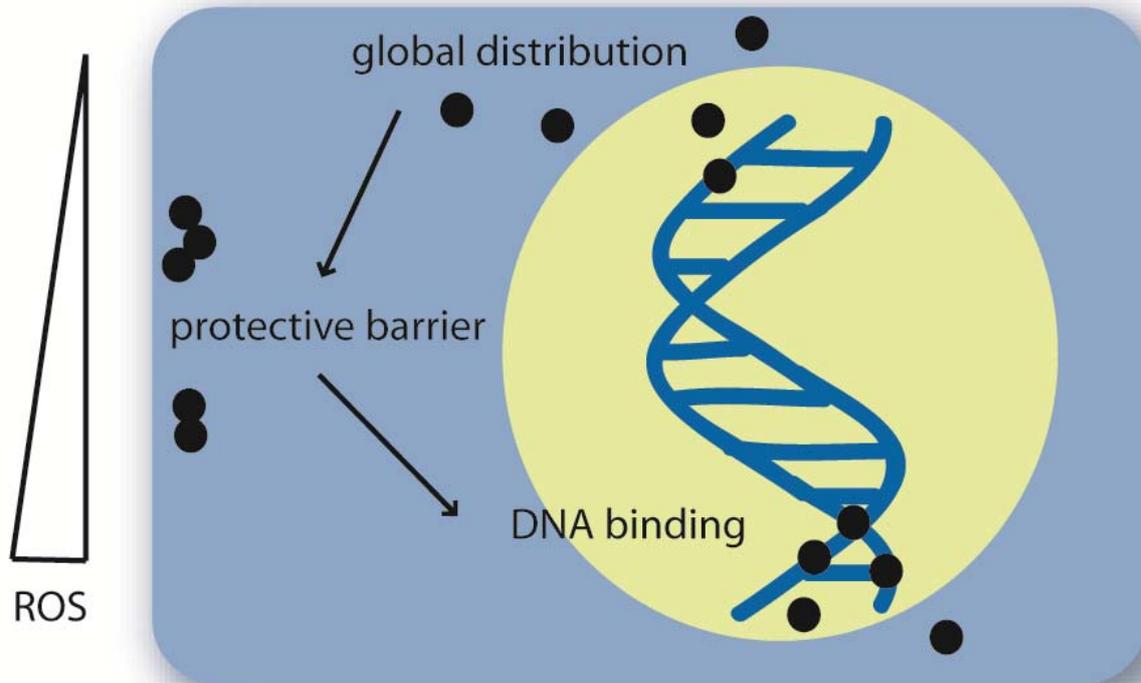
The largest GO cluster comprises multiple categories which can all be described under the denomination "nucleic acid binding" (cluster V in Fig. 1 and Table 1). Due to the large diversity of proteins in this category it is likely that the interaction between SPRR and these proteins is indirect and mediated by mutual interaction of each of these proteins with DNA. Since most GO categories of cluster V were more enriched among SPRR4 as compared to SPRR1B interactors, the cellular localization of SPRR4 was analyzed in order to reveal its potential nucleic acid binding capacity. Immunofluorescence staining with a SPRR4 monospecific antibody revealed minor but significant nuclear localization of SPRR4 proteins (Fig. 2A-C). In line with these results are earlier observations by other researchers who occasionally detected SPRR1, SPRR2, and SPRR3 proteins in the nucleus of cells by using various antibodies<sup>69,80,96,128,209</sup>. More recently, this was substantiated by live-cell-imaging of pEGFP-SPRR1B transfected keratinocytes where SPRR was consistently found in the nucleus of migrating cells<sup>192</sup>. This localization further highlights a potential role of SPRR proteins in DNA binding.

Since the above described SPRR localization studies as well as the GO data were obtained in the contexts of a whole cell, it was important to assess the DNA binding properties of purified SPRR proteins directly in an *in vitro* assay with purified components. Addition of increasing amounts of purified SPRR4 protein to linear DNA fragments indeed resulted in a lower mobility of the DNA molecules in agarose gel (Fig. 3A), which is a measure for direct interaction between proteins and nucleic acids. Purified SPRR1, SPRR2, and SPRR3 proteins showed similar electrophoretic mobility shifts, although a 10 times higher molar excess of protein was required (results not shown). Interaction of SPRR4 with plasmid DNA was also detected via AFM analysis. Here multiple small protein dots decorating the DNA molecules can be observed (Fig. 3C), suggesting that SPRR proteins bind DNA in a sequence independent way.

### ROS affects SPRR protein multimerization and DNA binding

Since the oxidation of SPRR proteins during ROS detoxification results in the reversible formation of both inter- and intramolecular S-S bonds<sup>191</sup>, we questioned whether these redox modifications can influence the DNA binding capacity of SPRRs. Analysis of the DNA binding activity of equal amounts of SPRR4, pre-treated with increasing concentrations of H<sub>2</sub>O<sub>2</sub>, revealed in a first instance a clear decrease in the DNA binding potential (Fig. 3D, lanes 1-5) suggesting an inverse relationship between SPRR protein multimerization and DNA binding. At higher H<sub>2</sub>O<sub>2</sub> concentrations, less protein multimers were observed on PAGE (Fig. 3E and F), while the DNA binding capacity increased. Mass spectrometric analysis of the different cysteine oxidation states revealed that at higher ROS levels disulfide bonds are broken by further oxidation to sulfinic- and sulfonic acid (Table 3), which provides an

explanation for a reversion to monomeric forms at higher H<sub>2</sub>O<sub>2</sub> concentrations. Apparently only monomeric SPRR4 can bind efficiently to DNA. At the highest ROS levels, however, DNA binding is again reduced whereas the amount of monomers still increases. This is likely due to the generation of cysteine sulfinate/sulfonate adducts, which might counteract DNA binding because of their higher negative charge.



**Figure 4.** Schematic representation of the impact of ROS on SPRR protein structure, cellular localization and molecular function. SPRR proteins are depicted by black disks. At normal ROS levels SPRR proteins are globally distributed within the cell both in nucleus and cytoplasm. At intermediate ROS levels, SPRR locates to the cell periphery and forms an efficient antioxidant barrier after multimerization due to cysteine oxidation while DNA binding decreases. At even higher ROS levels SPRR multimerization decreases and DNA binding increases again.

## Conclusion

Our data are summarized in a model illustrating how ROS affects the activity and cellular localization of SPRR proteins (Fig. 4). Under normal culture conditions, at low ROS exposure, SPRR proteins are globally distributed across the cytoplasm and nucleus of cells. In this position SPRRs directly provide protection against ROS induced DNA breakage. At intermediate ROS levels, cysteine residues become oxidized and SPRR protein multimers are formed via disulfide bonding. The cellular localization is then shifted towards the cytoplasmic membrane and the DNA binding activity is reduced. While localized at the cell periphery, SPRR proteins form an efficient protective barrier against cell-infiltrating ROS, which are often produced at the cytoplasmic membrane via lipid peroxidation following UV exposure<sup>46</sup>. In this respect it is important to note that SPRRs were originally identified as UV inducible genes<sup>86</sup>. If the ROS levels are too high and exceed the natural reducing potential of the cell, major oxidative stress arises and the cysteine residues of SPRR proteins become further oxidized. As a result, SPRR converts back to a monomeric form and the DNA binding activity increases again. Such high ROS levels will eventually lead to cell death, and many of the apoptotic signaling pathways will become activated by redox modifications<sup>28,197</sup>. However, since DNA itself is a very efficient ROS quencher, DNA binding by SPRR might temporarily delay DNA breakdown in order to provide antioxidant protection to neighboring cells. This compares to the normal situation in our skin where a layer of dead flattened cells protects the inner tissue against injurious external insults<sup>19</sup>. Apparently, the modulation of the oxidation state of SPRR proteins constitutes the basis for their selective antioxidant performance and fine-tunes their targeting to those cellular components that are most threatened at a given moment in time.

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## Summary and general discussion

The epidermis serves as a first line of defence against external insults, including toxic chemicals, bacterial/viral infections and solar UV radiation. A specialised structure within the cells of the outermost cornified layer, termed the cornified cell envelope (CE), is responsible for the mechanical and permeability properties of our skin. It consists of an extremely tough structure of cross-linked proteins and lipids, which still allows the high flexibility of our skin. Most of the identified CE proteins are transcribed from the epidermal differentiation complex (EDC), a 2.5 Mbp region on human chromosome 1q21. They include involucrin, loricrin, and the small proline-rich (SPRR) and LCE protein families. The head- and tail-domains of these CE precursor proteins are very similar and are utilised for transglutaminase cross-linking. The SPRR proteins are generally known as stress-inducible proteins. Following various forms of stress different SPRR family members are induced and subsequently modulate the skin's barrier function. In this way, the CE provides protection specifically adjusted to the type of damage involved.

All members of the SPRR gene family are tightly regulated at a transcriptional level. Each SPRR promoter sequence contains a complex panel of regulatory elements. In *Chapter II*, the biochemical cross-talk between the human Skn-1 isoforms is discussed. Two isoforms of this transcription factor, Skn-1a and Skn-1d1, were identified to bind the SPRR2A octamer site and activates its expression. Both isoforms bind the SPRR2A promoter region with similar affinity. Skn-1a functions cooperatively with Ese-1, an epithelial specific transcription factor previously shown to upregulate SPRR2A. However, this synergy was not found for Skn-1d1 and is apparently dependent on the extra N-terminal domain of Skn-1a. This differential cross-talk of the different Skn-1 isoforms plays an important role in the fine-tuning of SPRR gene expression during the cornification process and its adaptation to stress.

After disruption of the skin's barrier by wounding major stress arises after which the surrounding tissue has to react rapidly to restore the barrier, avoid infections and prevent loss of blood or tissue degradation. In *Chapter III* we analysed SPRR expression in response to wounding. At the edge of the wound massive SPRR expression was found that exceeds the normal expression in the differentiated skin layers. During the wound healing process reactive oxygen species (ROS) are generated as chemical steriliser against invading bacteria. In general, ROS are considered as toxic compounds as they can damage DNA, proteins, and lipids. As such, they are essential determinants of the ageing process and are involved in many human diseases, including Alzheimer, Parkinson, diabetes, and cancer. However, ROS are also naturally produced as signalling molecules which initiate the healing process. The local increase of ROS levels signal to the immune system to attract leukocytes. Subsequently, SPRR proteins directly reduce ROS levels via their cysteine residues. In this way, the tissue is protected from ROS induced damage but more importantly cell migration is allowed for proper wound closure. We identified SPRR as essential players in the wound healing process since its downregulation inhibited cell migration. This novel function is not only important following cutaneous wounding but plays a key role in all major tissues after various forms of injury, ranging from heart infarction to nerve regeneration.

Also non-wounded skin is continuously challenged by ROS. Solar UV radiation, toxic chemicals, air pollutants and bacterial/viral infections can all increase ROS. To cope with high ROS levels, the epidermis contains several cellular defence mechanisms. Multiple ROS-detoxifying enzymes, transcriptionally activated by Nrf2, and numerous low molecular weight antioxidants are gradually present in the different skin layers. During the cornification process, these groups of antioxidants are increased and subsequently intracellular ROS levels are reduced. Since SPRR proteins can actively reduce ROS, we questioned whether they could still fulfil this function after cross-linking within the CE (*Chapter IV*). Indeed, both purified SPRR proteins and CEs isolated from native skin significantly lowered ROS levels. Hence, the SPRR proteins within the CE constitute our first line of antioxidant defence. Upon oxidation SPRR proteins form both inter- and intramolecular disulfide bonds, which were also identified within the CE by mass spectrometry. Also the CE proteins filaggrin-2, KPRP, and loricrin were identified in this assay. Loricrin is a major component of the CE with a higher cysteine content than SPRRs. However, it contains a lower antioxidant potential compared to the individual SPRR proteins. Apparently, the number of cysteine residues per protein does not define its antioxidant potential which is more likely determined by structural differences. This also confirms the superior antioxidant properties of SPRR4. UV irradiation was previously shown to specifically induced SPRR4 expression. As a result, the CE properties are altered and the cornified layer is thickened. The upregulation of SPRR4 in response to UV results in adaptation of the skin's barrier and increases its antioxidant defence. These findings add, besides the mechanical and permeability properties of the CE, a highly adaptive and protective antioxidant shield to the skin's barrier.

In *Chapter V* we have examined the molecular mechanisms behind the protective antioxidant function of SPRR. Proteomic analysis of SPRR interaction partners identified known CE precursor proteins and proteins involved in the antioxidant response. This is in line with their established function in skin cornification and the newly identified protective function against ROS. Furthermore, proteins involved in cytoskeletal binding were identified, which reflects the function of SPRR in cell migration. Finally, multiple nucleic acid binding proteins were identified. DNA binding of SPRR, which occurs likely in a sequence independent manner, was confirmed and visualised by the use of several *in vitro* assays (bandshift analysis and AFM). Interestingly, ROS can modulate the SPRR protein activity and influence its DNA binding properties. Under normal conditions, at low ROS exposure, SPRR can bind DNA and prevent ROS-induced DNA damage. During oxidative stress, cysteine residues of SPRR become oxidised, protein multimerization occurs, DNA binding is reduced and its localisation is shifted from the nucleus to the cell periphery. While localising at the cytoplasmic membrane, SPRR proteins form a flexible antioxidant barrier, comparable to their role at the migrating front during wound healing. Apparently, modulating the oxidation state of SPRR proteins constitutes the basis for their global antioxidant performance. The skin's barrier can in this way be efficiently fine-tuned and adapted to specific tissue requirements, in order to provide optimal protection against ROS induced damage.





## Samenvatting

Elk organisme, van een vis tot een plant en van een bacterie tot de mens, heeft om te kunnen overleven een beschermende barrière nodig tegen invloeden van buitenaf. De epidermis is het buitenste deel van onze huid en is verantwoordelijk voor deze barrièrefunctie. De epidermis is opgebouwd uit meerdere lagen en biedt ons bescherming tegen o.a. mechanische stress, bacterie- en virusinfecties, schadelijke chemicaliën en uitdroging. Van binnenuit wordt de epidermis continu vernieuwd door delende, migrerende en differentiërende keratinocyten. Na de celdeling van een keratinocyte blijft er één cel achter in de basale, meest interne laag van de epidermis en begint de andere aan het cornificatieproces. Dit proces wordt ook wel terminale differentiatie genoemd, omdat de cel omhoog migreert via de bovenliggende huidlagen, sterk verandert van morfologie en eiwitsamenstelling, en eindigt als 'dode' huidschilfer in de hoornlaag. De "small proline-rich" ofwel SPRR-eiwitten spelen een belangrijke rol tijdens het cornificatieproces. Samen met enkele andere eiwitten worden zij aan de binnenkant van de celmembraan samengeketend tot een complexe stevige structuur, de zogenoemde "cornified cell envelope" (CE). Door de CE is onze huid sterk maar tegelijkertijd zeer flexibel. De expressie van de SPRR-eiwitten kan worden verhoogd door stress, variërend van zonlicht tot een huidinfectie. Dit resulteert in een toename van de hoeveelheid SPRR in de CE, en daarmee in een versterkte barrièrefunctie van de huid. De rol van de SPRR-eiwitten binnen het cornificatieproces alsmede de recentelijk gevonden expressie van SPRR-eiwitten in diverse weefsels en organen is beschreven in **Hoofdstuk I**.

Doordat diverse soorten stress de concentratie van specifieke SPRR-eiwitten kunnen reguleren, is er veel onderzoek gedaan naar de promotor-regio van de verschillende SPRR's. Elke SPRR-promotorsequentie bevat een complexe mix van verscheidene bindingsites voor transcriptiefactoren. In **Hoofdstuk II** is de regulatie van SPRR2A door de Skn-1 transcriptiefactoren onderzocht. Zowel Skn-1a als de verkorte isovorm Skn-1d1 bindt met vergelijkbare affiniteit aan de promotorsequentie van SPRR2A. Alleen de combinatie van Skn-1a met ESE-1, een tweede zeer bekende transcriptiefactor, zorgt voor een versterkte expressie van SPRR2A. De verschillen tussen de twee Skn-1 isovormen spelen een belangrijke rol bij de *fine-tuning* van de benodigde hoeveelheid SPRR tijdens het cornificatieproces en de adaptatie van de huidbarrière tegen stress.

Na verwonding van de huid is de beschermende barrièrefunctie sterk verzwakt. Dit resulteert in een enorme hoeveelheid stress in de huid, leidt mogelijk tot bloed- en weefselverlies en vergroot de kans op infecties. Het weefsel rondom de wond moet dan ook zeer snel reageren om de barrièrefunctie te herstellen. In **Hoofdstuk III** hebben wij de expressie van SPRR-eiwitten tijdens wondgenezing bestudeerd. Precies aan de rand van een wond was de hoeveelheid SPRR sterk verhoogd en was deze zelfs veel hoger dan in de normaal gedifferentieerde huid. Tijdens het genezingsproces worden direct na verwonding grote hoeveelheden zuurstofradicalen aangemaakt als chemische sterilisatie tegen bacteriën. Naast de zuurstof die wij inademen en nodig hebben om te overleven, bestaan er ook diverse reactieve vormen van zuurstof. Over het algemeen worden deze zuurstofradicalen gezien als zeer toxisch, omdat ze DNA, eiwitten en lipiden kunnen beschadigen. Als zodanig

zijn zuurstofradicalen een oorzaak van het verouderingsproces. Ook zijn ze direct betrokken bij diverse humane ziekten, zoals Alzheimer, Parkinson, suikerziekte en kanker. Echter, tijdens het begin van het genezingsproces spelen zuurstofradicalen ook een belangrijke rol bij de signaaltransductie naar het immuunsysteem en zorgen dat witte bloedlichaampjes (leukocyten) de exacte locatie van de wond weten te vinden. Vervolgens zorgen de SPRR-eiwitten, via enkele cysteïne-residuen, voor een verlaging van de hoeveelheid radicalen om zo het omliggende weefsel te beschermen tegen de schade ervan. Ook is deze verlaging noodzakelijk voor celmigratie en dus het dichten van de wond. De SPRR-eiwitten bleken essentieel te zijn in het genezingsproces, want wanneer we specifiek de hoeveelheid SPRR verlaagden, werd de celmigratie sterk geremd. Deze nieuw ontdekte functie van SPRR is niet alleen zeer belangrijk voor de huid, maar ook in alle andere weefsels na verschillende soorten letsel, variërend van regeneratie na zenuwschade tot het herstel na een hartaanval.

Ook zonder verwonding wordt de huid vaak blootgesteld aan zuurstofradicalen. O.a. zonlicht, schadelijke chemicaliën, luchtvervuiling, bacterie- en virusinfecties kunnen de productie van zuurstofradicalen induceren. Als bescherming bevat de epidermis diverse cellulaire afweermechanismen, waaronder verschillende enzymen en antioxidanten. Tijdens het cornificatieproces stijgt de expressie van beide groepen om de hoeveelheid radicalen te verlagen. Doordat we voor SPRR een vergelijkbare functie tijdens het wondgenezingsproces hebben gevonden, vroegen wij ons af of de verschillende SPRR-eiwitten ook als antioxidant kunnen werken terwijl ze vast geketend zijn in de CE (*Hoofdstuk IV*). Uit onze resultaten blijkt inderdaad dat zowel gezuiverde SPRR-eiwitten als ook uit de huid geïsoleerde CE's de hoeveelheid radicalen significant verlagen. De SPRR-eiwitten zorgen ervoor dat de buitenste laag van dode huidcellen zelf de eerste verdedigingslinie tegen oxidanten vormt. In hun antioxidantfunctie oxideren de cysteïne-residuen in SPRR en vormen ze inter- en intramoleculaire disulfidebruggen. Dit zijn omkeerbare verbindingen binnen een eiwitstructuur. Ook in de CE hebben we deze voor SPRR en enkele andere bekende CE-eiwitten teruggevonden. Eén ervan is loricrin, een belangrijk en veelvoorkomend eiwit in de CE. Het bevat meer cysteïnes dan de verschillende SPRR-eiwitten, maar toch is loricrin een veel zwakkere antioxidant. Blijkbaar is niet zozeer het aantal cysteïne-residuen van belang, maar wordt de antioxidant-werking bepaald door de verschillen in eiwitstructuur. Dit bevestigt ook de superieure antioxidantsterkte van SPRR4. In ons laboratorium is eerder aangetoond dat UV-bestraling zorgt voor een specifieke inductie van SPRR4-expressie. Als gevolg daarvan veranderen de eigenschappen van de CE en ontstaat er een verdikking van de huid. De inductie van SPRR4 na UV resulteert in een aanpassing van de barrièrefunctie van de huid en verhoogt de antioxidantcapaciteit ervan. Naast de mechanische- en permeabiliteitsfunctie van de CE voegen de SPRR-eiwitten ook een sterk adaptieve en beschermende antioxidantfunctie toe aan de barrièrefunctie van de huid.

In *Hoofdstuk V* zijn wij dieper in gegaan op het moleculaire mechanisme achter de beschermende antioxidantfunctie van de SPRR-eiwitten. Tijdens deze studie hebben wij diverse potentiële SPRR-bindende eiwitten geïdentificeerd. Enkele ervan spelen een bekende rol in het cornificatieproces of fungeren als antioxidant, wat in overeenkomst is met de bewezen functie in de huid van de SPRR-eiwitten, en met de recentelijk ontdekte beschermende rol tegen zuurstofradicalen. Verder zijn diverse cytoskelet-bindende eiwitten gevonden, wat past bij de rol van SPRR tijdens celmigratie. Ook zijn er meerdere

nucleïnezuur-bindende eiwitten geïdentificeerd. Aan de hand van deze resultaten voorspelden we dat SPRR mogelijk zelf aan DNA zou kunnen binden, wat inderdaad bevestigd werd door diverse experimenten. Ook vonden we dat de DNA-bindingscapaciteit van SPRR kan worden beïnvloed door zuurstofradicalen. Onder normale omstandigheden, bij een lage hoeveelheid radicalen, kan SPRR aan het DNA binden en voorkomt het schade aan DNA door zuurstofradicalen. Tijdens oxidatieve stress worden de cysteïne-residuen van SPRR geoxideerd en vormt het eiwit disulfidebruggen. De DNA-bindingscapaciteit wordt verminderd en de lokalisatie van SPRR verschuift van de kern naar de rand van de cel. Hier vormt SPRR een flexibele verdedigingslinie tegen zuurstofradicalen, die vergelijkbaar is met de lokalisatie van SPRR tijdens celmigratie bij het wondgenezingsproces. Blijkbaar vormt de oxidatietoestand van SPRR de basis voor de antioxidantfunctie ervan. Op deze manier kan ook de barrièrefunctie van de huid efficiënt worden aangepast om een optimale bescherming tegen zuurstofradicalen te bieden.

Al in 1851 heeft de Duitse professor Joseph von Gerlach bewezen dat de mens ook zuurstof via de huid kan opnemen. 160 jaar later, is het ons gelukt om voor het eerst de interactie tussen zuurstof en de menselijke huid op moleculair niveau te analyseren.



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## Curriculum Vitae

Wilbert Peter Vermeij was born on the 22<sup>th</sup> of March, 1980 in Gouda. In 1997, he graduated from the Senior General Secondary Education (HAVO) at the Coenecoop College, Waddinxveen. He went on to study Biotechnology at the Biological and Medical Laboratory Techniques in Delft, which later moved to Rotterdam. During his internship, he studied the expression levels of the human splice-variants of the p53-family members in skin cell-lines, under the supervision of Dr. M. Niemantsverdriet (department of Molecular Genetics, Leiden University). He obtained his Bachelor (Ingenieur) degree in August 2002. Between September 2002 and September 2004, he continued research in the group of Dr. Claude Backendorf and started working as a research technician. He combined his work for the Molecular Genetics department with his Chemistry study at the Leiden University. In 2003 and 2004, he completed two internships. The first one involved the preparation of fluorescent Annexin V for apoptosis studies in mammalian cells, under the supervision of Prof. Dr. J.F Nagelkerke (department of Toxicology, Leiden Amsterdam Center for Drug Research). Supervised by Dr. C. Backendorf, he studied RNA interference and reverse genetics in human and mouse keratinocytes. After completing this second internship he received the Master of Science degree in November 2004. In the same year, he started a PhD project termed "Antioxidant properties of small proline-rich proteins". This project was performed in the laboratory of Prof. Dr. J. Brouwer under the supervision of Dr. C. Backendorf (department of Molecular Genetics, Leiden University). The results of his scientific work are summarized in this thesis. Since May 2010, he was employed as Scientist at DNage B.V. The research, performed in Rotterdam and Leiden, involved both the identification of biomarkers and targets for specific ageing related diseases as well as the clarification of the mechanism of action of some lead compounds for the use as pharmaceutical or nutraceutical. Currently, he is employed as a post-doctoral researcher at the department of Genetics (lead by Prof. Dr. Jan Hoeijmakers) at the Erasmus Medical Center in Rotterdam where he is studying ageing related neurological disorders at a genomic and proteomic level.



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