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VIEWPOINT

pH sensing in skin tumors: Methods to study the involvement of GPCRs, acid-sensing ion channels and transient receptor potential vanilloid channels

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Abstract

Solid tumors exhibit an inversed pH gradient with increased intracellular pH (pH_i) and decreased extracellular pH (pH_e). This inside-out pH gradient is generated via sodium/hydrogen antiporter 1, vacuolar-type H + ATPases, monocarboxylate transporters, (bi)carbonate (co)transporters and carboanhydrases. Our knowledge on how pH_e-signals are sensed and what the respective receptors induce inside cells is scarce. Some pH-sensitive receptors (GPR4, GPR65/TDAG8, GPR68/OGR1, GPR132/G2A, possibly GPR31 and GPR151) and ion channels (acid-sensing ion channels ASICs, transient receptor potential vanilloid receptors TRPVs) transduce signals inside cells. As little is known on the expression and function of these pH sensors, we used immunostainings to study tissue samples from common and rare skin cancers. Our current and future work is directed towards investigating the impact of all the pH-sensing receptors in different skin tumors using cell culture techniques with selective knockdown/knockout (siRNA/CRISPR-Cas9). To study cell migration and proliferation, novel impedance-based wound healing assays have been developed and are used. The field of pH sensing in tumors and wounds holds great promise for the development of pH-targeting therapies, either against pH regulators or sensors to inhibit cell proliferation and migration.

KEYWORDS

cell migration, melanoma, skin barrier, squamous cell carcinoma, wound healing

1 | INTRODUCTION

Cancer remains one of the leading causes of death worldwide. Around 1.3 million cases of skin cancer (~7% of all cancer cases) were estimated worldwide for 2018, of which about 1 million were non-melanoma skin cancers (NMSC) and nearly 300 000 were melanoma.[1]

pH dysregulation is a hallmark of solid tumors, which further drives tumor growth, metastatic potential and immune escape.[2,3] The extracellular pH (pH_e) of solid tumor cells is more acidic (pH_e 6.2-7.0) as compared to normal cells (pH_e 7.2-7.4), while the intracellular pH (pH_i) of tumor cells (pH_i 7.2-7.7) is slightly increased compared to normal (pH_i 6.9-7.2).[4,5] This reversed/inside-out pH gradient is

thought to be responsible for some characteristic tumor cell behaviour, such as increased proliferation and migration or an altered immunological response. For example, in some studies extracellular acidification of the tumor environment increased the aggressiveness of melanoma cells by promoting their capacity to form metastases.[6]

However, in wounds these processes are important for healing, but somehow stopped along the way: sensors that detect changes in pH_e are potentially involved in feedback mechanisms that stop the necessary proliferation and migration of cells once a wound has healed. The striking similarities between wounds and tumors in terms of proliferation and migration led to Dvorak's description of tumors as wounds that do not heal.[7] Drastic changes in pH_e are observed during the wound healing process of cutaneous lesions. The physiological pH_e of normal healthy skin lays between 4 and 6, depending on anatomic localization and age, and plays an essential role for the homeostasis of the cutaneous barrier and regulation of the skin's microbiome.[8,9] Significant changes in cutaneous pH_e occur when the epithelial barrier is disrupted. The pH_e on the surface of acute wounds is in the range of pH 8.0-8.5 and within two weeks during the wound healing process, it gradually decreases to pH 6.0-6.5.[10] In contrast to physiological wound healing, chronic wounds exhibit lower pH_e at wound margins, which negatively influences central processes of wound healing like cell proliferation, centripetal cell migration, immune response and extracellular enzyme activity.[9-11]

The lower pH_e of solid tumors is, among others, caused by disorganized vascularization, the development of hypoxic regions, metabolic changes and an altered acid-base regulation[4,5] via the sodium/hydrogen antiporter (NHE1), the monocarboxylate proton symporters (MCT1, 2 and 4), vacuolar-type H^+ ATPases (V-ATPases), anion exchangers, (bi)carbonate (co)transporters and different carboanhydrases.[6,12] It is the adaption of cancer cells to extracellular acidosis that further drives tumor progression.

The cellular responses to dysregulated pH_e and pH_i are mediated by different cellular sensors. Intracellularly, many metabolic enzymes and cytoplasmic proteins are described as candidates that mediate pH_i-sensitive responses.[5] pH_e is likely sensed via cell surface receptors, such as proton-sensitive G-protein-coupled receptors (pH-GPCRs), transient receptor potential vanilloid channels (TRPVs) and acid-sensing ion channels (ASICs), that mediate the signals from the extracellular pH to the cell interior.[13]

Here, we focus on these types of sensors and show how we study their expression in skin. We also show concepts on how to analyse their impact on cell proliferation and migration in different skin cancers.

2 | PROTON-SENSITIVE GPCRS (PH-GPCRS): WHAT DO THEY DO IN (SKIN) TUMORS?

Proton-sensitive GPCRs (pH-GPCRs) are activated by a decrease of pH_e via the protonation of different histidine residues on the extracellular surface of the receptors.[14] The different pH-GPCRs show activation in a range between pH 5 and 8 with individual pH sensitivities and G-protein-coupled downstream pathways.[15-17] GPR4 (GPR19), GPR65 (TDAG8, T-cell death-associated gene 8), GPR68 (OGR1, ovarian cancer GPCR 1) and GPR132 (G2A, G2 accumulation protein) were initially described as receptors for different lysolipids,[17] before their sensitivity to changes in extracellular pH was demonstrated.[14,18,19] Recently, two more putative pH-GPCRs have been described, GPR31 and GPR151, for which studies on their physiological role in pH sensing are still rare.[15]

Changes in the expression of certain pH-GPCRs in tumor cells may be responsible for establishing an increased tumorigenic potential, as the pH-GPCRs were shown to play roles in tumor cell proliferation, apoptosis, metastasis, angiogenesis, immune cell function and inflammatory processes.[16,20] For example, GPR4, GPR65 and GPR132 were found to be often overexpressed in different cancer types, including tumors of the breast, ovary, colon, liver and kidney.[21,22] Indeed, ectopic expression of GPR4, GPR65 and GPR132 has been shown to induce malignant transformation of cultured cell lines.[21,23,24] On the other hand, pH-GPCRs such as GPR65 and GPR132 are widely expressed by cells of the immune system[16,17] and have been associated with tumor-suppressing functions.[25,26] Also for GPR68, counteracting effects have been described, depending on whether GPR68 is expressed in the tumor itself or in the cells of the host organism. GPR68 seems to function as a tumor-suppressor when expressed in the tumor itself with an inhibitory effect on cell migration and metastasis,[27-29] but can have tumor-promoting function being expressed in cells of the host organism.[30,31]

Concerning skin cancers, overexpression of GPR4 in squamous cell carcinoma has been shown to induce the production of cytokines and vascular endothelial growth factor (VEGF) which promoted angiogenesis, indicating tumor-promoting activity.[32] Another study showed that GPR4 overexpression in B16F10 melanoma cells inhibited acidic pH-induced migration, invasion and metastasis, pointing towards tumor-suppressing function.[33] GPR132 was found to be upregulated in human epidermal keratinocytes upon UVB radiation and H_2O_2 exposure and to be responsible for mediating lipid-induced cytokine production and cell cycle arrest,[34,35] thereby possibly functioning as sensor for DNA damage and oxidative stress and conveying tumor-suppressing activity.

Overall however, little is known about the expression levels and role of pH-GPCRs in skin cancers.[20] Therefore, we examined the expression profiles of pH-GPCRs in different skin cancers and performed immunohistochemical staining on tissue samples from different skin cancer types. We provided first evidence of pH-GPCR expression on the protein level in the selected rare non-melanoma skin cancers Merkel cell carcinoma (MCC), dermatofibrosarcoma protuberans (DFSP), atypical fibroxanthoma (AFX) and pleomorphic dermal sarcoma (PDS) in a previous publication in Experimental Dermatology.[36] Different expression patterns in the investigated skin cancer types indicate that the different pH-GPCRs may have distinct functions in tumor progression. For example, GPR4 and GPR65 were not expressed in MCC and also DFSP was negative for GPR65. In contrast, AFX and PDS tumors were positive for GPR4 and predominantly positive for GPR65. Many skin tumor types showed

expression of GPR132, while the expression of GPR68 appears to be very heterogeneous.

In our current work, we investigated the expression of pH-GPCRs in the non-melanoma cancers squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) as well as in malignant melanoma (MM) and (compound) naevus cell naevi (NCN). Our tissue microarray results show that the overall expression of all four GPCRs is increased in melanoma when compared to naevus cell naevi. In epidermal portions, this observation is even more pronounced (W. Klatt, S. Wallner, C. Brochhausen, J. A. Stolwijk, & S. Schreml, unpublished data). These results suggest that an increase in pH-GPCR expression in melanoma could be a marker for increased malignancy, which requires, however, further investigation.

The identification of characteristic expression patterns of the four different pH-GPCRs in different skin cancers may help to contribute to a better therapy. However, a deeper insight into detailed pH-GPCR function in tumor development and progression in different cell types is needed to address pH-GPCRs as therapeutic targets in skin cancer.

3 | ASICS AND TRPVS: ALSO PH SENSORS IN SKIN TUMORS?

Some pH-sensitive ion channels (acid-sensing ion channels ASICs, transient receptor potential vanilloid receptors TRPVs) transduce signals inside cells.[37] ASICs are non–voltage-gated cation channels transiently activated by a rapid drop in extracellular pH. Protons are the main physiological activators of these channels. The ASICs are selective for sodium and belong to the epithelial sodium channel (ENaC)/Degenerin family of ion channels.[38] TRPVs are non-selective cation channels with high Ca2 + permeability representing one out of seven subfamilies of the TRP channel superfamily.[39] The TRPV subfamily can be further subdivided into 6 isoforms—TRPV1-6. The varied distribution of TRPVs and their polymodal activation properties make them ideally suited to a role in perceiving and responding to local environmental changes. In humans, TRPVs are predominantly involved in sensing temperature and osmolarity. TRP channels overall have been reported to represent possible new targets for diagnosis and chemotherapy in cancer.[40] A recent review points out the emerging role of TRP channels in cancer progression.[41] The subfamily of TRPV channels has been mainly correlated with malignant tumor growth and progression by regulating cancer cell proliferation, apoptosis, angiogenesis, migration and invasion during tumor progression.[42] Interestingly, depending on the stage of the cancer, these aspects are associated with an increase or decrease in TRPV mRNA and protein expression.[43] A member of the TRPA subfamily, namely TRPA1, has been demonstrated to be activated by extracellular pH.[44] Moreover, TRPM7 channels show an increased channel activity as the extracellular pH decreases.[45] The most interesting candidates of the ASIC/TRPV ion channel families in terms of skin cancer are ASIC1/2 and TRPV1/4.[46-49]

As little is known about the expression and function of these pH sensors in tumors, we used immunostainings to study tissue samples from common skin tumors. First results from basal cell carcinoma (BCC), squamous cell carcinoma (SCC), naevi (NCN) and melanoma (MM) are shown (Figure 1). Studies in a larger number of samples will now be performed to answer questions regarding expression profiles of these pH sensors.

4 | OPEN QUESTIONS AND EXPERIMENTAL APPROACH

Expression studies in different skin tumor tissue give an idea of the prevalence and local distribution of the putative pH sensors pH-GPCRs, ASICs and TRPVs. The expression of ASICs and TRPVs has not been studied in detail for the various types of skin tumor. It is also not known how the levels of expression of pH-GPCRs, TRPVs and ASICs influence cancer cell behaviour. Not only pH sensing of the tumor cells, but also the pH response of the surrounding tissue seems relevant for tumor progression and remains to be investigated. Identifying the relevant pH sensors and their influence on tumor progression of different tumor types may help to develop individual therapeutic strategies. Therefore, we aim at investigating the role of these pH sensors in cell migration, proliferation and immune modulation, which are central functions of tumorigenesis. After identifying individual protein levels in different cell types (qPCR, Western blot), the next step is to use knockdown (siRNA)/knockout (CRISPR/Cas9) and overexpression strategies in combination with functional cellular assays to answer these questions. The next paragraph describes a non-invasive, automated assay to study cell migration and proliferation, which we use to investigate pH-dependent behaviour of skin cancer cells.

5 | MIGRATION AND PROLIFERATION IN IMPEDANCE-BASED ASSAYS

In order to study the migration of different cultured skin cancer cell types (eg SCC, BCC, melanoma), we use an impedance-based, automated wound healing assay (Figure 2). The assay relies on growing the cells to confluence on thin gold-film electrodes deposited on the bottom of a cell culture dish. Cell coverage of the electrodes is monitored by non-invasive impedance readings.[50,51] As the adherent cells act like insulating particles and block current flow, the impedance gradually increases with increasing cell coverage. Though in principle, different electrode dimensions and geometries can be used for impedance-based assays, a small working electrode in combination with a significantly larger counter electrode are typically used for wound healing assays.[52] After baseline recordings, the cells on the small electrode are killed by applying invasive AC currents of 2.4 mA amplitude at 32 kHz frequency for 30 s inducing irreversible membrane electroporation (Figure 2A).[53,54] Cell wounding is indicated by a clear drop

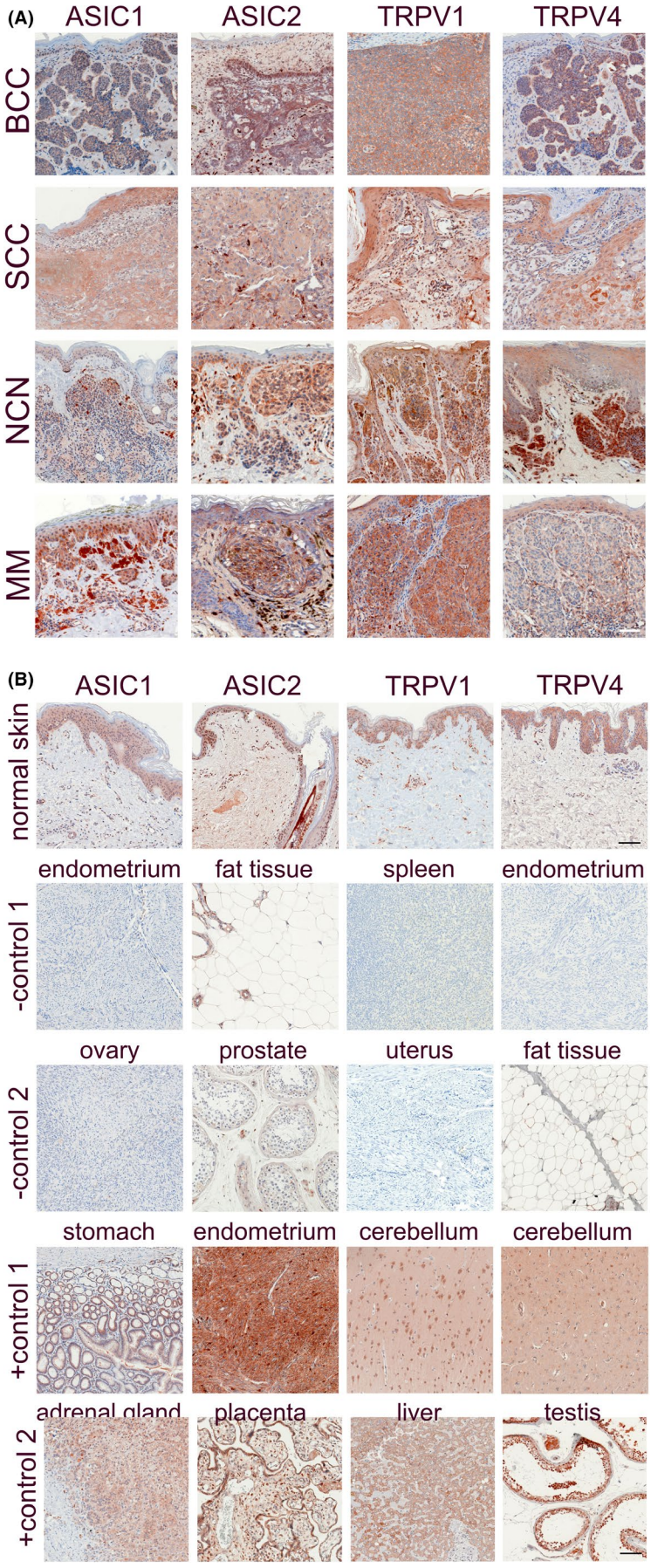

FIGURE 1 Immunohistochemistry of ASIC1/2 and TRPV1/4: (A) Representative samples of the most common skin tumors, that is basal cell carcinoma (BCC), squamous cell carcinoma (SCC), naevus cell naevus (NCN) and melanoma (MM) were stained for ASIC1, ASIC2, TRPV1 or TRPV4, according to the protocol as described in Nassios et al[36] and using the following primary antibodies: ASIC1: PA5-26778, ASIC2: PA5-26222, TRPV1: LS-B12677 (Thermo Fisher Scientific Inc, Waltham, MA, USA), TRPV4: ab21912 (abcam, Cambridge, UK). B, Normal skin (first row) and control tissues (second to fifth row) were stained for ASIC1, ASIC2, TRPV1 or TRPV4. Negative (second and third row) and positive (fourth and fifth row) controls were selected according to expression analyses published at the human protein atlas (https://www.proteinatlas.org/). Scale bars: 100 μm. Compared to epidermis, BCC showed strong expression of the four pH-sensitive proteins. In contrast, SCC showed a quite similar expression of all pH-sensitive proteins in comparison to normal epidermis, markedly weaker than BCC. For NCN and MM, epidermal and dermal expression was quite different. NCN exhibit strong expression of ASIC1/2 in the more superficial portion, while the intensity decreases in dermal melanocytes. However, TRPV1/4 seems to be uniformly expressed even in deeper tissue layers. In MM, ASIC1/2 and also TRPV1 are also expressed throughout the whole tumor. In contrast, TRPV4 staining showed only weak expression in MM, which may serve to distinguish NCN from MM

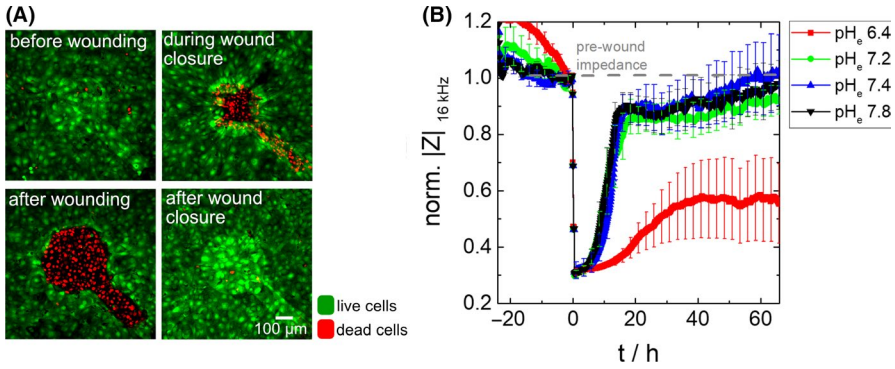

FIGURE 2 Impedance-based migration assay: (A) Live/dead staining of squamous cell carcinoma cells (SCC111, DSMZ) grown onto thin gold-film electrodes before wounding, immediately after electric wounding, during wound closure and after migration-based wound healing. Cells on the electrodes were stained with the LIVE/DEAD Viability/Cytotoxicity Kit (Thermo Fisher Scientific Inc) and inspected by confocal fluorescence microscopy. Viable cells appear green, and nuclei of dead cells are stained red. B, Analysis of pH-dependent migration of SCC111 cells grown on gold-film electrodes. Prior to the experiment, the cells were grown to confluence in electrode arrays under normal cell culture conditions. After 48 h of preculture, regular medium was replaced by serum-free and (bi)carbonate-free Leibovitz L-15 medium adjusted to four different pH values (6.4, 7.2, 7.4 and 7.8), respectively. After 24 h of adaptation to the different pH values at ambient CO_2 levels, the cells were wounded by the electrical pulses (32 kHz, 2400 µA for 30 s) and the time-dependent impedance recovery was recorded. Impedance was normalized to the final value before pulse application. Averaged impedance values immediately prior to wounding at time zero were pH 6.4: (9.0 ± 1.2) kΩ, pH 7.2: (9.4 ± 1.2) kΩ, pH 7.4: (9.2 ± 1.8) kΩ and pH 7.8: (9.3 ± 1.4) kΩ. Data represent averages with their corresponding standard error (mean ± SE, $n \geq 4$) acquired from at least four individual runs. $T = 37°C$. Impedance data were recorded using the ECIS™Zθ system (Applied BioPhysics Inc). Fluorescence micrographs (A) were taken for cells grown on 96-well electrode arrays (Type 96W1E+, Applied BioPhysics Inc), while impedance time courses (b) were recorded for the same cells grown on 8-well electrode arrays containing a small working electrode ($5 \cdot 10^{-4}$ cm^2) and a much larger counter electrode (A = 0.75 cm^2) in each well (Type 8W1E, Applied BioPhysics Inc)

in impedance down to values of a cell-free electrode (Figure 2B). Cells on the large counter electrode or next to the electrodes are not affected by the pulse and migrate onto the electrode from the periphery, thereby successively replacing the dead cells (Figure 2A). The concomitant impedance recovery reports on the time-dependent repopulation of the electrode so that analysis of the time course data allows an assessment of cell migration under different experimental conditions (Figure 2B).

For example, Figure 2B shows the migration of cultured squamous cell carcinoma cells (SCC111) cells as a function of extracellular pH. The time course of the impedance magnitude (|Z|) has been recorded at an AC frequency of 16 kHz for confluent SCC111 cell layers at four different extracellular pH values before and after electrical wounding at time zero. Data represent the mean of at least four individual experiments. The impedance magnitude is normalized to the last |Z|value before wounding. Immediately after wounding and membrane permeabilization, the impedance drops to normalized values of 0.3 representing impedance values close to the values of cell-free electrodes, as the permeabilized cell bodies no longer restrict current flow. After a short lag phase, the impedance starts to recover at individual rates reporting on the time course of cell migration. For pH values close to the physiological pH of 7.4 (pH 7.2 & 7.8), the time-dependent impedance recovery after electric wounding is very similar and does not indicate any pH-dependent impact on cell migration. The prepulse impedance values are largely recovered within 16 hours indicating complete wound closure. In contrast, a more acidic pH (pH 6.4) led to a significantly delayed and incomplete recovery of the impedance. Accordingly, the capability of SCC111 cells for collective cell migration is significantly affected by acidic pH in the extracellular environment.

Compared to many other assays used for analysis of cell migration, the impedance-based assay allows for a time-resolved and automated data recording and the electrical wounding provides very precise wounds that are solely defined by the dimensions of the small electrode. As impedance measurements are sensitive to the amount of cells that populate the electrode, impedance-based assays may also be used to study cell adhesion or cell proliferation as a function of different pH conditions.[52] Future studies aim at investigating the role of pH-sensing proteins using knockdown/knockout or overexpressing cell models in these assays.

6 | PERSPECTIVES

In this article, we focus on the pH-sensitive GPCRs and the ion channels ASICs and TRPVs. Other possible interesting pH-sensitive "candidates" for further studies in investigating the role in pH sensing in skin tumors are represented by members of the two-pore domain (K2P) K^+ channel family. Some ion channels of this family—the TASK channels (two-pore domain, acid-sensitive K^+ channels)—are sensitive to changes in extracellular pH in the physiological range, making them likely candidates to mediate various pH-dependent processes.[37] For TASK-3, high expression levels were reported in melanoma cells and channels affected apoptosis and mitochondrial function in these cells.[55,56]

Once relevant pH sensors are identified, targeted strategies for clinical settings (antibodies) may be developed that extend the few existing approaches to control tumor pH.[20] Since tumors and chronic wounds show many parallels, future wound management therapies may also benefit from emerging findings.[9,11,20] In addition to refined in vivo pH-detection technologies, wound dressings with pH-dependent drug release or targeting pH-dependent signalling pathways may be developed.[11,57] New wound dressing materials (eg based on nanofibers) with improved capabilities for integrated pH sensing and drug release are currently being developed.[58,59]

In the long run, a basic understanding of the pH sensing and control mechanisms in skin tumors and chronic wounds is inevitable for improved therapies. The identification of characteristic expression patterns of the different pH-sensing proteins in different skin cancers and the investigation of their role in proliferation and migration may help to contribute to a better therapy.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

JS, LS, JW and SS conceptualized experiments. JS, LS and KA performed research. AN, TA, SH and A.B contributed thoughts to the paper. JS and SS wrote and compiled the text.

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