Chapter 6

General discussion
Skin wound infections with antimicrobial-resistant bacteria are considered one of the greatest threats to public health worldwide. The rapid emergence of antibiotic-resistant bacteria has been attributed to the adaptation of these microorganisms to hostile environmental conditions, as well as the overuse and misuse of antibiotics. As a result, skin wound infections are highly challenging to treat, often resulting in morbidity and mortality due to the development of septic processes in the patients. Novel approaches on a non-antibiotic basis are therefore urgently needed to fight bacterial resistance and prevent infections in skin wounds. This thesis focuses on the development and validation of a fibrin-based human skin equivalent (HSE) with overexpression of the antimicrobial peptide LL-37, and the optimization of their mechanical properties for skin tissue engineering applications.

THE CHOICE OF THE GENE DELIVERY SYSTEM

As a first step, we needed to choose a gene delivery system that would allow the genetic modification of primary keratinocytes and fibroblasts with the precursor gene that leads to LL-37 production (i.e. CAMP gene). It has been described that the success of gene therapy is largely dependent on the development of a vector or carrier that can efficiently deliver a gene to target cells with minimal toxicity. Two widely used systems for nonviral gene delivery are polyplexes and nucleofection. Nucleofection toxicity in human keratinocytes has been previously reported, with results that showed a decrease in cell size and with only 20%-42% of proliferative cells. This coincided with our findings in primary keratinocytes, since nucleofection led to a significant decrease in cell viability, although with higher transfection efficiencies in both keratinocytes and fibroblasts. Decreasing the cytotoxicity of nucleofection on keratinocytes implies optimization of the buffers purchased from the manufacturer, which in addition to increasing costs is quite challenging since information on the components of all solutions is lacking. The polyplexes, on the other hand, proved to be efficient enough to achieve a high number of copies of the CAMP gene with low cellular toxicity. Furthermore, polyplexes can be prepared in a few steps using inexpensive reagents and can be stored for a longer time than nucleofection solutions. This cost-benefit ratio is especially important when conducting research in developing countries.
Transfection efficiency is commonly considered as the key factor when comparing different transfection methods. However, few studies consider the influence of the sequence to be introduced into the target cells, which in most cases is a plasmid vector. Our plasmid vector contained an internal ribosome entry site (IRES) to allow separate expression of the reporter gene (red fluorescent protein) and the CAMP gene in target cells, since the expression product of CAMP (i.e., LL-37) is metabolized extracellularly. We demonstrated that the expression of the reporter gene located downstream of the IRES sequence and whose translation was IRES-dependent was reduced compared to the absence of IRES (chapter 2). Similar results were described in previous studies carried out in bicistronic plasmids, which, like ours, contained two distinct genes within one vector. Nevertheless, most recent studies found that gene expression under the control of multiple IRES elements has no effect on the posttranscriptional regulation in multicistronic plasmids, and that the fluorescence output of reporter gene is proportional to the number of IRES repeats. Therefore, care should be taken regarding the construction of the plasmid vector, as well as in determining which gene should be positioned as the first or second gene in a bicistronic construct. In our case, the CAMP sequence was located upstream of IRES, so its translation was IRES-independent.

Based on our findings, the polyplexes system was chosen for the genetic modification of skin cells in this work. Since the construction of HSEs suitable for tissue engineering applications must start from healthy cultures, mainly healthy keratinocytes capable of proliferating and forming stratified epithelia similar to native skin, and capable to produce larger amounts of the LL-37 peptide together with fibroblasts.

LINEAR POLY(ETHYLENIMINE) (LPEI): A BETTER ALTERNATIVE FOR SKIN CELL TRANSFECTION.

Branched and linear forms of poly(ethylenimine) (BPEI and LPEI, respectively) have been commonly used as gene therapy delivery agents. The success of these polymers in gene delivery strictly depends on the kind of target cells, and until now, their behavior in the transfection of primary fibroblasts and keratinocytes had not been described (chapter 3). BPEI and LPEI in different weight concentrations were varied for evaluating the formation and colloidal characteristics of the polyplexes. The PEI based polyplexes showing diameters between 250 and
450 nm and a positive surface charge (+30 ± 2 mV) at a Nitrogen/Phosphate ratio of 19, show the desired characteristics for interaction with the cell membranes and subsequent endocytosis. This positive surface charge confers electrostatic stability to the solution and prevents polyplexes aggregation since it allows sufficient repulsive forces between them.19

Several studies have reported that the transfection efficiency of LPEI polyplexes in vitro was greater than that of BPEI polyplexes.20 They also proposed that this behavior might be a result of a less strong conjugation of LPEI with the DNA. In transfected keratinocytes and fibroblasts, both LPEI and BPEI allowed the expression of the reporter gene and increased the CAMP gene expression. However, LPEI showed superior performance as it led to higher transfection efficiencies with the highest cell viabilities compared to BPEI. This is due to the fact that LPEI formed less compacted polyplexes than BPEI, so it is possible that they can dissociate easily within cells and release DNA, while polymer residues are degraded or diluted by exocytosis processes.21

These findings contribute to a broader knowledge of the effects of polyplexes system on the genetic modification of skin cells and allowed to establish the optimal transfection conditions for both human keratinocytes and fibroblasts.

THE GENERATION OF HSEs WITH ANTIMICROBIAL POTENTIAL

Even though various human skin substitutes are commercially available (chapter 1), to date no dermo-epidermal model for the prevention of skin wound infections has been reported. In this work, we developed fibrin-based HSEs seeded with primary fibroblasts and keratinocytes previously transfected through LPEI polyplexes (chapter 4). Prior to the construction of the HSEs, quantification of the LL-37 peptide in monolayer cultures of transfected cells showed a low production of the peptide despite the fact that these cells expressed high copies of the CAMP gene. This result was initially considered contradictory since it was expected that the increase in the number of copies of the gene was accompanied by equal changes in the encoded proteins. However, this is not always the case. In fact, how cells change the expression of proteins in response to their environment is one of the most fundamental questions in biology. The multitude of steps between transcription and translation provides many different regulatory opportunities and always leaves a “door open” for future research questions.22,23
Taking this into account, we challenged the transfected cells through stimulation with metabolites derived from bacteria, obtaining a significant increase in the LL-37 peptide production compared to the non-transfected cells. The increase in LL-37 levels was also observed in HSEs constructed with these modified cells and remained elevated up to 14 days in culture. Furthermore, HSEs containing transfected cells were able to decrease bacterial growth of a planktonic strain of *S. aureus* after 24 hours of co-culture.

The increased secretion of the LL-37 peptide after the transfected HSEs were exposed to metabolites from bacteria correspond with what has been reported about the expression of this peptide on epithelial surfaces, especially for keratinocytes in airways or skin. Under normal conditions, the *CAMP* gene product is directed to the storage granules of cells and can be stimulated by both exogenous microbial components or endogenous signal molecules such as lipopolysaccharides or bacterial DNA.

In this sense, the lack of stimulation could explain the results obtained in the *in vitro* antimicrobial activity tests when the LPEI/pDNA and BPEI/pDNA polyplexes were compared (chapter 3). It was observed that supernatants from the cells transfected with BPEI did not show activity against the evaluated bacterial strain, despite the fact that the expression of the *CAMP* gene had previously been confirmed. But, based on the findings in chapter 4, it is possible that LL-37 was produced but remained in storage. Although a deep understanding of the mechanism by which polyplexes act was not an objective of this thesis, our observations indicate that not only the transfection efficiency determines which is the ideal vector, but also the processes that continue after the expression of the gene of interest and the type of protein it encodes.

In addition to the antimicrobial potential, the expression of LL-37 in HSEs was shown to be cytocompatible toward human keratinocytes and fibroblasts, promoting cell proliferation (chapter 4). This finding was in line with most of the previous literature reports and could be explained due to the association of LL-37 to cell receptors like the fibroblast growth factor receptor (FGFR) and the epidermal growth factor receptor (EGFR). However, a recent study in which the *in vitro* cytocompatibility of LL-37 with stem cell metabolism and chondrogenic differentiation was investigated, reported that concentrations above 25 μg/ml were
toxic and caused cell death in more than 70%. Thus, it is important to determine the optimal concentration of LL-37 in relation to the cell type for future research.

The expression of LL-37 in HSEs also did not affect the formation of the dermo-epidermal compartments, which showed morphological characteristics similar to normal skin. These characteristics together with the antimicrobial properties are promising for the therapeutic use of our skin model since it has advantages compared to the use of antibiotics, due to the natural difficulty that microorganisms have to acquire resistance to the host defense peptides such as LL-37. However, care must be taken since the first bacterial resistance against LL-37 has been developed in *Escherichia coli* strains.

**OVERCOMING THE MECHANICAL LIMITATIONS OF HSEs**

Fibrin is a biopolymer of interest in tissue engineering for building HSEs that can be used in wound healing. It can be easily obtained from the patient’s blood, which makes it a suitable alternative in clinical protocols for autologous use. However, after a deformation process, fibrin loses high amounts of water, which subsequently makes it mechanically unstable and, finally, difficult to manipulate.

Although the mechanical stability of HSEs should not interfere with the secretion of the LL-37 peptide and therefore with their antimicrobial activity, it is a condition that must be improved in order to broaden their clinic applications. The combination of fibrin with other natural polymers such as agarose is considered a novel strategy to improve the overall biomechanical and structural properties of the fibrin while supporting cell functions. The viscoelastic properties and response of human dermal fibroblasts to fibrin-agarose (FA) hydrogels produced with varying concentrations of agarose showed that the addition of agarose increased the stiffness of the FA hydrogels and prevented the contraction of the matrix, which could solve one of the limitations for handling, transport, implantation and performance of these hydrogels (chapter 5). Besides, the viscoelastic properties of FA hydrogels were strongly correlated with the increase in agarose concentration, and in turn, these properties affected cell behavior, mainly cell proliferation and metabolism. Recent studies have also found a potential impact of the hydrogel viscoelasticity on cellular behaviors, such as cell spreading, proliferation, and differentiation. The role of time-dependent mechanics on cell biology remains largely unclear and ripe for
further exploration. The extracellular matrix (ECM) has been shown to be a key regulator of tissue mechanics, which in turn affects various aspects of cell behavior. This has typically been studied in the context of purely elastic matrices and it is unclear how cells interpret these signals in the context of viscoelastic matrices. Cells generate forces and deformations on substrates in a highly dynamic manner. These interactions lead to a complex cellular response that regulates cellular gene expression (mechanotransduction). Therefore, tools and approaches that allow deciphering cell-matrix interactions with greater spatiotemporal resolution are needed, e.g. super-resolution imaging in three dimensions, molecular force sensors, and materials with dynamically tunable mechanical properties, which could address this need providing detailed information on the interactions and dynamic forces that occur between cells and viscoplastic matrices.

Further elucidation of this topic could substantially advance our understanding of cell-matrix interactions and guide the design of improved biomaterials for regenerative medicine, such as HSEs increasingly similar to native skin.

This thesis also demonstrated the importance of interdisciplinary research to propose novel alternatives with a view to improving people’s quality of life. The development of HSE with antimicrobial properties was achieved by coupling tissue engineering with disciplines such as gene therapy and biomaterial science. Thanks to the combination of these disciplines, most of the objectives proposed in this work were achieved. However, there are still some questions to be resolved: 1) will HSEs have a similar antimicrobial activity against other bacterial strains? 2) could LL-37 secretion contribute to wound healing? By solving these questions, the clinical applications of the model could be broadened to prevent infections in skin wounds.

In addition, the HSEs could be evaluated in combination with 1% agarose to improve its mechanical properties and evaluate its effects on cell proliferation and metabolism. Furthermore, since the connection between matrix viscoelasticity and cell signaling could activate transcription factors, which in turn could regulate protein expression, it will be interesting to evaluate the effects of viscoelastic properties of HSEs on LL-37 expression.
REFERENCES

16. Wojtowicz, A. M. et al. The importance of both fibroblasts and keratinocytes


31. Chaudhuri, O., Cooper-White, J., Janmey, P. A., Mooney, D. J. & Shenoy, V.