Reporter System Controlled by the Involucrin Promoter as a Tool to Follow the Epidermal Differentiation

Myrian Thiago Pruschinski Fernandes
 University of Sao Paulo (EACH-USP)

Jeniffer Farias dos Santos
 University of Sao Paulo (EACH-USP)

Bruna Letícia Freitas
 University of Sao Paulo (EACH-USP)

Gustavo Roncoli Reigado
 University of Sao Paulo (EACH-USP)

Fernanda Antunes
 University of São Paulo School of Medicine (FM-USP)

Nayara Tessarollo
 University of São Paulo School of Medicine (FM-USP)

Felipe Santiago Chambergo
 University of Sao Paulo (EACH-USP)

Bryan Eric Strauss
 University of São Paulo School of Medicine (FM-USP)

Viviane Nunes (✉ vanunes@ib.usp.br)
 University of Sao Paulo (EACH-USP)

Research Article

Abstract

Different approaches have been explored to study skin biology, including the use of stem cells. Mesenchymal stem cells (MSC) from umbilical cord can be safely and easily obtained, however a simple strategy to monitor their differentiation is essential. Involucrin is a marker of keratinocyte terminal differentiation, and its promoter (pINV) directs stratum-specific expression of this protein. We designed a reporter system containing EGFP under control of pINV to assess MSC differentiation into keratinocytes. The functional sequence of pINV was inserted into a lentiviral vector, originating LeGO-GpINV. MSC were transduced with the LeGO-GpINV and induced to differentiate into keratinocytes upon cultivation with Keratinocyte Serum Free Medium supplemented. MSC differentiation was confirmed by morphological changes and by the expression of epidermal markers, by flow cytometry, quantitative PCR and western blot. The activity of kallikreins 5, 6 and 7 was detected using fluorogenic substrates. After 14 days of differentiation, MSC transduced with LeGO-GpINV showed an increase in EGFP fluorescence and expressed CK10, CK14, involucrin and filaggrin. There was also an increase in the kallikrein activity. This reporter system allowed to temporally assess the epidermal differentiation, simultaneously with involucrin expression, opening perspectives for the *in vivo* study of skin biology and in regenerative medicine.

Introduction

The skin is the major organ in the human body, covering and protecting it against external aggressors. The outermost layer of the skin is the epidermis, which is responsible for the barrier function, preventing the water loss and the access of toxins and pathogens [1]. The epidermis is formed by four strata or layers. The basal stratum is the germ layer in which keratinocytes, the main cell of the epidermis, proliferate and migrate to the upper layers while undergoing complex morphological and biochemical changes in approximately 28 days. These changes constitute the process of epidermal differentiation and are coordinated by the expression of specific genes [2].

During the terminal differentiation of keratinocytes there is intense proteolytic activity in which the combined action of different enzymes, such as the epidermal kallikreins (hK) 5 and 7, results in the desquamation process of skin and loss of the nucleus and other cellular organelles. Other proteins such as cytokeratins (CK) 1, 5, 10 and 14, involucrin, filaggrin, loricrin and p63 also participate in the keratinocyte differentiation process [3]. They present specific expression in the strata of the epidermis, being used as markers of epidermal differentiation. Involucrin, a 68 kDa protein, is considered one of the key markers of terminal differentiation of keratinocytes, whose expression begins in the suprabasal layers [4].

Skin injuries or some pathological conditions may compromise the skin barrier, exposing the organism to external insults. Despite the properties of healing and wound repair, such as severe burns and diabetes, the skin cannot regenerate [5, 6]. Therefore, it is essential to study the molecular mechanisms involved in skin differentiation, both in physiological events and in pathological conditions, in order to meet the

emerging demand for developing solutions related to skin in regenerative medicine, tissue bioengineering, gene therapy and *in vitro* models [7] with high interest for the use of stem cells.

Different sources of stem cells have been explored in strategies for skin repair and substitution. Mesenchymal stem cells (MSC) from umbilical cord are adult stem cells that can be obtained by a non-invasive method, which is an advantage when compared to other sources of adult stem cells [8, 9]. In addition, they are less immunoreactive [10] and it has been shown that they have immunosuppressive, anti-inflammatory, and anti-proliferative properties [11]. However, monitoring the differentiation of these cells in specific cell types remains a challenge [12] and the existing methods do not allow monitoring of the process over time [13].

A successful strategy to monitor the differentiation of stem-cell in other lineages is the observation of reporter gene expression directed by promoters that regulate the transcription of specific genes involved to differentiation [14, 15]. Different methods of transfection have been explored in recent decades, including gene transfer with viruses, which are considered very effective for delivery and integration of genes in MSC. The most used viral vectors in gene transfer are those based on lentiviruses, adenoviruses, baculoviruses and adenoassociated virus [16]. Nunes et al.[9] demonstrated that stem cells obtained from umbilical cord blood transduced with lentivirus achieved an efficiency in transduction of more than 70% of the cells.

Recent studies have investigated the use of specific gene promoters controlling reporter gene expression to monitor lineage differentiation [13, 14]. Different regulatory promoters of epidermal differentiation proteins have been explored to monitor the differentiation of keratinocytes, such as the CK1 or CK14 promoters [17, 18]. However, since involucrin present suprabasal expression, its promoter (pINV) appeared as a more suitable candidate to identify the terminal differentiation of these cells. Another advantage of pINV is that involucrin is expressed in epidermal cells even under conditions in which other markers are not well expressed [19, 20, 21].

Considering the potential for the use of MSC in therapeutic applications and in the development of epidermal substitutes, we designed a reporter system containing the fluorescent reporter gene EGFP (enhanced green fluorescent protein) under control of pINV to monitor the differentiation of MSC into keratinocytes in a time-spatial manner.

Results

Obtaining the involucrin promoter

The functional regions of the involucrin promoter, RRP and RRD, with 242 bp and 404 bp, respectively, were amplified from genomic HaCaT DNA, as shown in **Figure 1A (Supplementary Figure 2)**. The amplified RRD and RRP segments were ligated, at the *Bam*HI restriction site, generating a 659 bp

fragment, which corresponds to the pINVf (**Figure 1B**). The pINVf primers were designed with specific restriction sites to facilitate cloning (**Figure 1C**).

Construction and analysis of the recombinant vector

After the construction of the LeGO-GpINV vector (**Figure 1D**), two strategies were used to confirm the replacement of pSFFV by pINVf. Using the *LEGO-F* and *R* primers in PCR experiments, the amplification of a 726 bp fragment from the LeGO-G/NeoOpt (control) indicated the presence of pSFFV, and a fragment with 850 bp in LeGO-GpINV (recombinant vector) indicated the presence of pINVf. When the primers *INV-F* and *R* were used, a 659 bp fragment was amplified confirming the presence of pINV in the LeGO-GpINV. In the control vector, there was no amplification, indicating the absence of pINVf (**Figure 1E**). According to the digestion strategy, the LEGO-G/NeoOpt vector digested with both *Nhe*I and *Eco*RI enzymes released a fragment with 1960 bp, corresponding to a sequence containing pSFFV, while the recombinant vector was only linearized, indicating absence of pSFFV (**Figure 1F**).

An 850 bp fragment amplified from LeGO-GpINV was sequenced. The resulting sequence from the amplification with the primer *LEGO-R* (**Supplementary Figure 1**) showed 100% identity in the alignment with the predicted sequence. The restriction sites *Xho*I, *Bam*HI and *Asc*I were identified, as well as the essential binding sites (AP1-1, AP1-5, C/EBP and SP1) for involucrin expression, and the cis-acting TATA box region. Replacement of pSFFV with pINVf was also confirmed, and its in-phase insertion and location upstream of EGFP. As the expression of the EGFP reporter protein in LeGO-GpINV is conditioned to the transactivation of pINV during involucrin expression, concentration of the lentiviral production was inferred from LeGO-G/NeoOpt titration by flow cytometry. The titer of the viral preparation was 5.67×10^8 TU/ml (transducing units per ml).

Characterization of MSC

Cultured MSC were characterized by flow cytometry using anti-CD105, anti-CD73 and anti-CD90 antibodies, which recognize surface markers present in these cells. As negative controls, MSC were incubated with anti-CD34 and anti-CD31 antibodies (**Figure 2**), surface antigens present in hematopoietic and endothelial stem cells, respectively. About 99.3% of cells were positive for CD90, 99.6% for CD105 and 98.9% for CD73. MSC incubated with anti-CD34 and CD31 antibodies were not labeled, with only 0.2% and 0.4% of positive events, respectively. HaCaT cells were incubated with CD90 and CD105 (**Figure 2B**) and a minimum percentage was labeled, 0.5% and 0.3%, respectively, which confirms that the markers are specific to MSC.

Differentiation of MSC into keratinocytes

For the study and standardization of methods related to the expression of epidermal differentiation markers, preliminary assays with non-transduced MSC were performed. When cultured in the proliferation medium, MSC possesses a fibroblast shape (**Figure 3A**). After 7 days of cultivation in KSFM supplemented with 1.8 mM calcium ions and 5 ng/ml EGF, the cells acquired a polygonal shape and were juxtaposed (**Figure 3B, C, D**). Furthermore, it is possible to visualize cellular filaments, which, although not further investigated, resemble actin filaments (indicated by black arrows).

Expression of epidermal differentiation proteins by flow cytometry

The control and differentiated cells were incubated with the specific antibodies for CK10, CK14 and involucrin and analyzed by flow cytometry (**Figure 4**). All antibodies were efficient for labeling the proteins of interest. On MSC differentiated for 14 days, more than 85% of the cell population expressed CK14, CK10 and involucrin. For CK10, it was possible to identify an increase in the population of cells expressing CK10, as well as in the fluorescence intensity, between days 7 and 14. For involucrin, there was an increase in the population of cells expressing involucrin on the seventh day of culture. There was no labeling on the first day of differentiation or for cultivation with the proliferation medium (negative control). For HaCaT cultivated in DMEM (positive control), 89.4% of the cell population expressed involucrin, 81.2% CK10 and 97.9% CK14.

Detection of the specific activity of epidermal kallikreins

There was a significant and progressive increase in the specific activity of kallikreins in MSC grown with the differentiation medium compared to those cultured in DMEM (negative control) on the same day (**Figure 5A**). For hK5, the difference was verified for day 1 and for hK6 and 7, the difference was observed on the seventh day of cultivation. Kallikrein activity was significantly lower in MSC grown with DMEM and this activity did not change during the time course of cultivation. In differentiated cell cultures, hK5 activity increased 1.8 times on day 14 compared to day 1. For hK6 and 7, the difference occurred between all periods, being 2.0 and 2.6 times higher at day 14 compared to the first day. In order to ensure that the enzymatic activity observed in the differentiated MSC was related to the presence of epidermal kallikreins, assays were performed with specific inhibitors for each protease studied. The hK5 activity was completely inhibited by 100 and 500 µM SBTI and 100 µM aprotinin (**Figure 5B**). This figure also shows that hK7 activity was almost 50% inhibited by 500 µM TPCK.

Expression of involucrin and filaggrin by qPCR

The expression of involucrin and filaggrin in MSC differentiated into keratinocytes was also studied by RT-qPCR (**Figure 6A**). When MSC were grown with the differentiation medium, the relative expression of involucrin increased significantly on day 7, being 4.2 and 5.1-fold higher on days 7 and 14, respectively,

compared to the first day of cultivation. When cells were grown with DMEM, there was no increase in the expression during the analyzed period. In differentiated MSC, filaggrin relative expression was 10-fold higher on the seventh day of culture when compared to the first day, with no significant increase between days 7 and 14, nor in cells cultured in DMEM.

Involucrin expression by western blot

The expression of involucrin was higher in MSC cultured in KSFM on days 7 and 14 in comparison to cells cultured in DMEM on the same days (**Figures 6B, 6C, Supplementary Figure 3**). There was a significant increase in the expression of involucrin in MSC on the 14th day of differentiation compared to the seventh day, which was about 27.2 times higher than in DMEM in the same period. As positive control, HaCaT cells cultured in both media were analyzed on the seventh day of the cultivation period, with a 10-fold increase in the involucrin expression in HaCaT cells cultured with KSFM.

Transduction of epidermal cells with lentiviral vectors

The cells were transduced with viral particles produced from LeGO-GpINV and analyzed by inverted fluorescence microscopy on days 0, 1, 7 and 14 of differentiation. MSC induced to differentiate into keratinocytes showed a gradual increase in fluorescence on days 7 and 14 of the differentiation process. In MSC growth with DMEM (day zero) or non-transduced cultivated with KSFM (control), there was no fluorescence (**Figure 7**). The increase in EGFP expression on days 7 and 14 of culture confirms that the expression of the reporter protein was transactivated by pINVf and that this promoter causes tissue and stratum-specific expression.

The expression of the reporter protein in the transduced MSC was also evaluated by flow cytometry. When transduced with the recombinant lentiviral vector, MSC showed an increase in the population of fluorescent cells on the seventh day of culture with KSFM. The intensity of fluorescence due to EGFP expression was also analyzed, being verified the presence of a population of cells that express this protein with high-intensity fluorescence (36.7%) on the seventh day of culture, compared to lesser than 5% in cells cultured in DMEM or transduced with the control vector (**Figure 7D-II**). When transduced with the LeGO-G/NeoOpt vector (MOI of 4), MSC cultured in DMEM (day 0) had 74.62% of cells expressing EGFP, indicating the effectiveness of transduction with the original vector.

HaCaT cells were also transduced to confirm the expression of EGFP controlled by the involucrin promoter in cells that endogenously express the involucrin protein. These cells were cultured in DMEM or KSFM supplemented with 1.8 mM calcium ions and 5 ng/ml EGF, being observed an increase in the population of cells expressing the reporter protein on the seventh day of culture with the differentiation medium. In HaCaT cells cultured in DMEM, there was no difference in the population of cells expressing EGFP (75%) between cells transduced with LeGO-G/NeoOpt or LeGO-GpINV.

Discussion

Different approaches have been explored to monitor and to understand the molecular mechanisms involved in the epidermal differentiation process of MSC into keratinocytes for both therapeutic and biotechnological applications [22, 23]. In the present study, we designed a reporter system controlled by pINV as a tool to continuously monitor the epidermal differentiation of MSC.

Before differentiation and transduction experiments, MSC were characterized by immunophenotyping. According to the International Society for Cellular Therapy [24], more than 95% of the MSC population must express CD73 (ecto-5'-nucleotidase), CD90 and CD105. Our samples resulted in approximately 99% of the cells expressing these markers. CD34, a hematopoietic marker, and CD31 (PECAM-1: platelet adhesion molecule), an endothelial marker, were used as negative controls [25]. Specifically, less than 1% of MSC expressed the negative markers CD31 and CD34.

We have already shown that it is possible to obtain cells with characteristics of keratinocytes by the two-dimensional cultivation of MSC in KSFM in the presence of 1.8 mM calcium ions and supplement containing EGF 5 ng/ml, being verified the expression of epidermal markers in a process temporally consistent to one that occurs in human skin [26]. Accordingly, MSC cultivated in these conditions showed typical morphological changes after the seventh day of cultivation, such as changing the fibroblastoid to polygonal shape like the morphology of keratinocytes in cultures [27]. In addition, it was possible to observe structures that resemble actin filaments, characteristic of suprabasal keratinocytes [28].

The current method to induce epidermal transdifferentiation of MSC resulted in an expressive population of cells expressing CK14, CK10 and involucrin after 14 days of culture (higher than 85%). The high expression of involucrin and filaggrin after 14 days of differentiation also indicate that most cells were terminally differentiated.

Among the several factors that influence the proliferation and differentiation of keratinocytes [29], the concentration of calcium ions has a great influence on the regulation of epidermal differentiation and the expression of different epidermal markers, such as involucrin and hK [30]. One of the pathways for regulating the expression of epidermal differentiation markers is the PKC pathway, which is strongly activated by calcium. The extracellular calcium gradient increase leads to the enhancement of transcription factors ligation to its respective binding sites present in pINV [31]. However, there are controversies in the action of EGF to induce epidermal differentiation [29, 32].

In addition to demonstrating the ability of MSC to transdifferentiate into keratinocytes, it was essential to show the expression of involucrin in cells induced to differentiate simultaneously with the expression of the reporter protein. In MSC induced to differentiation, increased expression of involucrin was identified on days 7 and 14 of culture and filaggrin on day 7. In contrast, in cells cultivated in the proliferation medium, there was no expression of these proteins. By flow cytometry, approximately 100% of MSC cultured with differentiation medium, for 7 days, were labeled with anti-involucrin antibody. This

expression persisted, being identified on the 14th day of cultivation, which indicates that most of the cell population reached terminal differentiation. These data agree with previous studies [5, 6, 26] and corroborate the effectiveness of the proposed protocol to differentiate MSC in keratinocytes.

Several authors have already differentiated MSC in epidermal cells under different culture conditions and evaluated the involucrin expression, which indicates the terminal differentiation. However, our method showed very effective compared to other studies [6, 33, 34], obtaining a high population of cells expressing involucrin after 14 days and higher relative expression of involucrin mRNA. In the present study, the expression of involucrin and filaggrin mRNA was 5 and 10 times higher, respectively, after 14 days of culture with differentiation medium when compared to cells cultured in DMEM. When evaluated by western blot, the expression of involucrin in differentiated MSC was 27 times higher than in cells cultured in DMEM in the same period, and about 3 times higher than in HaCaT cells cultured in this medium.

In addition to the assessment of epidermal marker expression, MSC differentiation into keratinocytes was evaluated by the detection of the specific enzyme activity of tissue kallikreins on the hydrolysis of specific FRET substrates. Tissue kallikreins are considered attractive targets for the development of new therapies involving the airways, structures of the cardiovascular system, teeth, brain, skin and neoplastic diseases [35]. Also, recently, they appear as innovative targets for the treatment of some skin diseases, such as Netherton's syndrome [36], with different approaches being proposed to detect and inhibit their activity. The tissue kallikrein activity measured in cell extracts was compatible with those of Santos et al. [26], with a progressive increase in the activity on days 7 and 14 of cultivation of MSC with the differentiation medium. Additional assays were performed using specific inhibitors of trypsin and chymotrypsin-like activity, and the activity of hK5 e 7 was inhibited by the tested inhibitors, according to Goettiga et al. [37].

In this study, we established a monitoring system that allows us to trace the differentiation of MSCs into keratinocytes by EGFP expression under the control of pINV. Therefore, that tool will allow monitoring the differentiation in a temporal, accurate and easy way, evaluating the effect of different chemical and/or physical stimulation in the epidermis. The pINVf was successfully amplified and cloned, maintaining the AP1-1, AP1-5, C/EBP and SP1 binding sites, as confirmed by sequencing, which are essential for the involucrin expression [31, 38, 39]. Comparing to other epidermal promoters, such as the pCK14 [40], pINVf has been proved to be a good candidate for viral vector production, since promoters larger than 1.5 Kb fail to deliver tissue-specific genes and can result in reduced viral titer and is able to promote high expression of the reporter protein [17].

Different studies have shown that lentiviral is efficient as a gene delivery method in MSC transduction. Nunes et al. [9] achieved a transduction efficiency greater than 70% in MSC from umbilical cord blood with lentivirus encoding EGFP. Zhang et al. [41] demonstrated that MSC from bone marrow transduced with lentivirus showed a proportion of transduced cells expressing the EGFP and DsRed reporter proteins higher than 75% using MOI below 1.0 and persistent expression for more than 5 months. It has also been

shown that viral transduction of MSC can reach up to 90% of cells, without affecting the quality of progeny and the differentiation capacity of these cells [42, 43].

In the LeGO-GpINV construction, the antibiotic resistance (neomycin) and the reporter gene are downstream from pINVf, which restrict its transactivation to cells that express involucrin and not in non-differentiated cells. Therefore, the titration and the MOI assays was performed with the LeGO-G/NeoOpt preparation and the viral titration and MOI was extrapolated to the recombinant vector preparations. Although, our results demonstrated that HaCaT cells transduced with LeGO-GpINV had similar transduction efficiency to the HaCaT cells transduced with LeGO-G/NeoOpt vector.

Both cell types were transduced in suspension with MOI 4 with concentrated viral preparation and the efficiency of approximately 75% of cells expressing EGFP was achieved in MSC transduced with the LeGO-G/NeoOpt vector and in HaCaT cells transduced with LeGO-G/NeoOpt or LeGO-GpINV. In addition, more than half of the differentiated MSC population expressed EGFP on the seventh day of culture, compared with 75% of positive cells in the transduction of MSC with the LeGO-G/NeoOpt vector.

Accordingly, it was shown an increase in the green fluorescence intensity corresponding to the expression of EGFP on days 7 and 14 of differentiation of MSC transduced with LeGO-GpINV. In parallel, it was verified, by flow cytometry, an increase in the population of cells expressing involucrin on day 7. In non-differentiated MSC transduced with LeGO-GpINV, the expression of the reporter protein was not detected, confirming the specificity of the expression of EGPF. These results demonstrate that pINVf was able to promote stratum-specific expression of EGFP, simultaneously with the expression of involucrin in these cells. The results obtained corroborate with previous studies [6, 44, 45], where it was demonstrated that the involucrin promoter is active in the cells that express this protein.

Collectively, the results show that KSFM supplemented with calcium and EGF ions was efficient to induce the transdifferentiation of MSC into keratinocytes and that these cells showed expression of differentiation epidermal markers over 14 days of cultivation. It was also verified that the EGFP expression coincides with the expression of involucrin in these cells, demonstrating that the tool is functional and efficient to monitor the terminal differentiation of keratinocytes. In summary, the reporter system proved to be very promising in a differentiation model of MSC in keratinocytes *in vitro*. This tool can be extended to 3D skin models, opening perspectives to be used as a simple and low-cost option to monitor cells *in vivo*. In addition, it can enable a better understanding of skin biology, both in physio and pathological conditions or for MSC use in tissue bioengineering and in regenerative medicine.

Methods

Construction of recombinant vectors

To obtain the involucrin promoter, the RRD and RRP functional regions of pINV, as previous described [17, 38], were amplified using oligonucleotides synthesized with specific restriction sites, as shown in **Table 1,**

from the genomic DNA of HaCaT cells, extracted with QIAamp DNA Stool Mini Kit (QIAgen, Hilden, Germany). The minimal functional involucrin promoter (pINVf), a 659-bp segment, was obtained through ligation of RRD and RRP in *Bam*HI restriction site, followed by amplification. The amplifications were performed by polymerase chain reaction (PCR) using Taq Platinum kit (Invitrogen, Waltham, MA), following the program: 95ºC for 5 min; 35 cycles of 95ºC for 30 sec, 55ºC for 30 sec, 72ºC for 1 min; 72ºC for 10 min.

Table 1. Oligonucleotides

FRAGMENT	POSITION	OLIGONUCLEOTIDES	OLIGONUCLEOTIDES SEQUENCE
RRD 404-bp	-2473/ -2069 pb	*INV-F*	5' CCG**CTCGAG**AAGCTTCTCCATGTGTCATG 3'
		INV-Rint	5' CGC**GGATCC**GGGTTAGCAGGGGCTCAGTA 3'
RRP 242-bp	-242/ -1 pb	*INV-Fint*	5' CGC**GGATCC**CCTAGGTCAGAAAAGCATCAC 3'
		INV-R	5' TT**GGCGCGCC**GAGCTGAGCAGGAGTCAG 3'
Construction Checking		*LEGO-F*	5 'GTAATTGTGTGTTTTGAGAC 3'
		LEGO-R	5 'GAACTTGTGGCCGTTTAC 3'

* The restriction sites are indicated in bold

The pINVf segment was inserted into the lentiviral vector LeGO-G/NeoOpt [46] between the *XhoI* and *AscI* restriction sites, upstream the EGFP, replacing the spleen focus-forming virus (pSFFV) virus promoter. OneShot® Stbl3TM (Invitrogen, Waltham, MA) competent *E. coli* were used for vector DNA amplification.

To confirm the correct construction and absence of pSFFV in the vector, two PCR amplification reactions were performed using two sets of primers (**Table 1**): 1) *LEGO-F* and *LEGO-R*, which are located upstream and downstream of the pSFFV sequence, respectively; and 2) *INV-F* and *INV-R*. The presence of the pINVf sequence and the absence of pSFFV was also confirmed by digestion of the vectors with the enzymes *Eco*RI and *Nhe*I, considering that the *Nhe*I site is present only in the vector LeGO-G/NeoOpt, upstream of the pSFFV. The DNA sequencing covered the flanking regions of the pINVf insertion site in the lentiviral vector. A fragment was amplified using the primer set *LEGO-F* and *LEGO-R* (**Table 1**). Subsequently it was sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA) and DNA sequencing reaction was carried out in an ABI 3730 DNA Analyzer (Applied Biosystems, Waltham, MA) by capillary electrophoresis using the Sanger sequencing method [47].

Lentiviral packaging

Lentiviral particles were packed using a 2nd generation packaging system. Plasmids LeGO-G/NeoOpt, pCMV-VSVg envelope and psPAX2 were mixed, and transfection of HEK 293T cells (2×10^6) was performed using the calcium phosphate precipitation method, adapted from Naviaux et al. [48]. Lentiviral supernatants were collected 24 and 48 h after transfection, filtered through a 0.45 μm pore size syringe filter and then concentrated by ultracentrifugation at 110,000 x g for 90 min at 4ºC in the SW 32 Ti Rotor (Beckman Coulter, Brea, CA). The pellets were resuspended in 100 μl of Hank's Balanced Salt Solution (HBSS - Life Technologies, CA) and incubated at 4ºC for 24 h. Solutions were stored at -80ºC. The lentivirus titration was performed in HT1080 cells (5×10^4), a human fibrosarcoma cell line. The cells were incubated with the viruses for 6 to 8 h and subsequently the expression of the reporter protein was analyzed by flow cytometry in a FACSCALIBUR cytometer (Becton Dickinson, Franklin Lakes, NJ) at λ = 530 nm. The results were obtained in TU/ml (transducing units per ml) and virus titers > 10^6 per ml (for non-concentrated supernatants) were obtained, as expected [49].

Cell cultures

MSC from the umbilical cord were obtained as described by Santos et al [26], as approved by the Research Ethics Committee of Federal University of Sao Paulo (3301230915) HaCaT cells, a commercially available lineage of spontaneously transformed and non-tumorigenic keratinocytes [50], were used as controls in the different experiments. These cells are part of the cell bank of Laboratory of Skin Physiology and Tissue Bioengineering, School of Arts, Sciences and Humanities, University of Sao Paulo. Cells were cultivated in the proliferation medium (Dulbecco's modified Eagle's medium, DMEM), with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 10,000 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen, Waltham, MA), in a humidified atmosphere and 5% CO_2 at 37°C. MSC were used in the passages from 2 to 10 as indicated in the different experiments.

Characterization of MSC by flow cytometry

MSC immunophenotyping was performed according to the expression of cell surface markers detected by flow cytometry. Five thousand cells per sample were evaluated on the Guava EasyCyte™ system (Merck Millipore, Burlington, MA) using a blue argon laser (488 nm) and readings were performed at λ=525/30 nm, λ=583/26 nm and λ=695/50 nm. The cells were incubated with the primary antibodies conjugated with different fluorophores: anti-CD90-FITC, anti-CD105-PE, anti-CD73-PE, anti-CD34-PerCP and anti-CD31-PE (Abcam, Cambridge, MA), for 1 h. As negative control, cells were stained with anti-mouse IgG-FITC (Invitrogen, Waltham, MA) or anti-human IgG-PE (Santa Cruz Biotechnology, Dallas, TX) secondary antibody, for 1 h. The results were expressed in terms of the percentage of labeled cells in relation to the total number of events.

In vitro differentiation of MSC into keratinocyte-like cells

MSC were distributed in 6-well plates and cultured in proliferation medium until reaching 80% confluence. Induction of MSC to differentiate into keratinocyte lineage was performed by culturing in Keratinocyte Serum Free Medium (KSFM) (Gibco, Grand Island, NY) supplemented with 1.8 mM calcium ions and the specific supplement Defined Keratinocyte-SFM Growth Supplement containing 5 ng/ml EGF. The cells were analyzed before changing the culture medium to KSFM (day zero) and after 1, 7 and 14 days of culture with this medium. The morphological changes were accompanied by optical microscopy in a Nikon Eclipse TS100 inverted microscope (Nikon Instruments Inc., USA).

Expression of epidermal differentiation markers

The expression of epidermal differentiation markers was evaluated by flow cytometer, qPCR and western blot in MSC induced to differentiation after different incubation times (0, 7 or 14 days). HaCaT were used as positive control.

Flow cytometry. The cells were pelleted, fixed and permeabilized using the Fix & Perm Cell Fixation and Cell Permeabilization kit (Thermo Fisher Scientific, Waltham, MA) and stained with the primary antibodies: anti-involucrin (Invitrogen, Waltham, MA), anti-CK10 (GeneTex, Irvine, CA) and anti-CK14 (Merck Millipore, Burlington, MA). The secondary antibody anti-IgG-Alexa Fluor 488 was added for 1h. As negative control, cells were stained only with secondary antibodies. The evaluation was performed by flow cytometry in the Guava cytometer, obtaining 5,000 events per sample. The results were analyzed according to the percentage of labeled cells relative to the total of events.

Western blot. The cultivated cells were disrupted with lysis buffer (25 mM HEPES, pH 7.5, 0.5% Triton X-100, 2 mM EDTA), containing the SIGMAFAST™ Protease Inhibitor Cocktail (Sigma Aldrich, San Luis, MI) according to the manufacturer's recommendations. Samples were centrifuged at 10,000 x g for 5 min for debris removal. Protein quantification was performed according to the Bradford method [51]. A total of 25 µg protein-aliquots were dried in a dry bath, resuspended in 15 µl of sample buffer (BoltTM LDS Sample Buffer, Bolt^{TM} Sample Reducing Agent - Novex, Invitrogen, Waltham, MA) and heated at 95ºC for 5 min. The soluble proteins were resolved by SDS-PAGE (5 or 12% gel) and transferred on to nitrocellulose membranes. The non-specific sites of the membrane were blocked in a blocking buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.10% Tween 20 containing 5% skimmed milk), for 1 h. The membrane was reacted overnight at 4ºC with the primary antibodies anti-involucrin (0,75 µg/ml; Thermo Fisher Waltham, MA) and anti-β-actin (1:2000; Abcam, Cambridge, MA). Blots were incubated for 1 hour at room temperature with secondary antibodies anti-mouse IgG-HRP (1:1000; Abcam, Cambridge, MA) and anti-rabbit IgG-HRP (1:1000; Santa Cruz Biotechnology, Dallas, TX) and immunoreactive bands were detected with chemiluminescent detection reagent Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA) on the ImageQuant ™ LAS 500 equipment (Cytiva Life Sciences, Marlborough, MA). Densitometric analysis was performed using the GelAnalyzer 19.1 software (www.gelanalyzer.com). The

results were normalized in relation to the expression of β-actin. The relative expression was obtained in, at least, four different experiments.

Quantitative PCR (qPCR). Cellular extracts from HaCaT cells were used as positive control and MSC cultured in DMEM and RINm5F cells, a cell lineage derived from rat insulinoma, were used as negative controls. Total RNA from the cultured cells was extracted using Brazol reagent (LGC Biotecnologia Ltda, Cotia, SP). The cDNA was obtained by reverse transcription using the QuantiNova™ Reverse Transcription kit (QIAgen, Hilden, Germany). The amplification of the involucrin and filaggrin gene segments was performed with the SYBR® green PCR Master Mix reagent (Thermo Fisher Scientific, Waltham, MA), in the Eco™ Real-Time PCR System (Illumina Inc, San Diego, CA) with the following cycling conditions: 50ºC for 2 minutes; 95ºC for 10 minutes; followed by 40 cycles of 95ºC for 30 sec, 60ºC for 30 sec and 72ºC for 1 minute. To standardize the amount of RNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control. The following oligonucleotides were used: Involucrin-F 5' AATGAAACAGCCAACTCCACTGCC, Involucrin-R 5' TCTTGCTTTGATGGGACCTCCACT, Filaggrin-F 5' GTTACAATTCCAATCCTGTTGTTTTC, Filaggrin-R 5' CGTTGCATAATACCTTGGATGATC, GAPDH-F 5' CAACCTAACAACTGATAATGCC, GAPDH-R 5' GTCTTTCATTTCCTCCTCGT. Relative mRNA expression levels were measured by the $2^{-\Delta\Delta Ct}$ method. At least three different biological experiments performed with technical duplicates.

Enzyme specific enzyme activity of epidermal kallikreins

The MSC cultured for 1, 7 or 14 days in the proliferation or differentiation medium were lysed with 300 μl of the lysis buffer (25 mM HEPES, pH 7.5, 0.5% Triton X-100). HaCaT cells were used as positive control. The cell extracts were centrifuged at 10,000 x g for 10 min, at 4ºC, and the supernatants were used to detect hK5, -6 and -7 specific activity on the hydrolysis of the fluorogenic substrates (FRET, Fluorescence Resonance Energy Transfer) Abz-KLRSSKQ-EDDnp, Abz-AFRFSQ-EDDnp and Abz-KLYSSKQ-EDDnp, respectively. The substrates were synthesized and kindly provided by Prof. Dr. Maria Aparecida Juliano from the Biophysics Department of the Federal University of São Paulo. Reactions were performed using 40 μl of cell extracts, 50 μl of the Enzyme Reaction Buffer (100 mM Tris, pH 8.0, 300 mM NaCl and 0.01% Tween 20) and 10 μl of each substrate (final concentration 10 μM). The hydrolysis of the substrates (10 μM) was followed by the increase in the fluorescence at λ_{ex}=320 nm and λ_{em}=420 nm per minute in the Synergy HT plate reader (BioTek Instruments, Winooski, VT), corresponding to the reaction mean rate. The enzyme activity was expressed as arbitrary units of fluorescence/min per 1 μg of protein in the cell extracts determined according to Bradford method [51]. Four different experiments, at least, were carried out in duplicates. The enzyme activity was characterized using specific inhibitors 100 and 500 μM TPCK (Tosyl phenylalanyl chloromethyl ketone, a chymotrypsin-like activity inhibitor), 100 and 500 μM SBTI (Soybean trypsin inhibitor, a trypsin-like activity inhibitor), and 100 μM aprotinin, a serine protease-like activity inhibitor [37]. The results were expressed as the residual enzyme activity, according to the percentage of inhibition in comparison to the control sample.

Transduction of cells with lentiviral vector

The transductions were performed in MSC at the 2 and 3 passages, and HaCaT as positive control, at 80% of confluence. The cells were detached by trypsin and the lentiviral preparation at a multiplicity of infection (MOI) 4 were added in the cell suspension with DMEM containing 10% SFB and the presence of 8 µg/ml of Polybrene (Sigma Aldrich, San Luis, MI). The cells plated to the final concentration of $5x10^4$ cells/ 600 µl of medium per well and incubated for 6 to 8 h. After, 1 ml of the medium was added, and the cells were incubated again. After 24 h, the medium was replaced by DMEM containing 10% SFB. Cells were analyzed by flow cytometry in a FACSCALIBUR cytometer (Becton Dickinson, Franklin Lakes, NJ).

Reporter protein expression by fluorescence microscopy

The analysis of the transduced cells was performed 10 days after the transduction, to discard any signal of reporter protein due to a nonintegrated vector. The cells were analyzed during proliferation and after induction of epidermal differentiation, on days 1, 7 and 14. The expression of EGFP was investigated by fluorescence microscopy using the Zeiss AxioObserver D1 Inverted Fluorescence Microscope (Zeiss, Germany). The green fluorescence detection was carried out at a wavelength of 470 nm. The images were captured using the software AxioVision4.8 (Zeiss, Germany), with a 100-x magnification. The experiments were performed at least three times and the images were analyzed using the ImageJ software (https://imagej.nih.gov/ij/). The mean fluorescence intensity per pixel was determined and the threshold of 10 defined as background.

EGFP expression by flow cytometry

The expression of EGFP in the differentiated MSC (or HaCat cells as positive control), was assessed by flow cytometry in a Guava flow cytometer, as previously described. The cells were pelleted and resuspended in 300 µl of PBS. 5,000 events were acquired per sample. The expression of the reporter protein was evaluated in terms of the percentage of cells with fluorescence in relation to the total of events.

Statistical Analysis

The data were expressed as the means ± standard deviation and analyzed by two-way analysis of variance followed by Bonferroni post-test, for multiple comparisons, using GraphPad Version Prism 5 (GraphPad Software Inc., San Diego, USA). Differences were considered statistically significant when $p<0.05$. The statistically significant differences were evaluated comparing the cells cultivated in DMEM or KSFM, on the same day (**, $p<0.01$; ***, $p<0.001$) or comparing the days of cultivation with the same treatment (#, $p<0.05$; ##, $p<0.01$; ###, $p<0.001$).

Declarations

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Author statement:

Myrian Thiago Pruschinski Fernandes – myrianpf@gmail.com – Investigation, Formal analysis, Data Curation, Writing - Original Draft

Jeniffer Farias dos Santos - jeni.fs@hotmail.com – Methodology

Bruna Letícia Freitas - bruna.leticia.freitas@usp.br – Investigation

Gustavo Roncoli Reigado - gustavoroncoli@hotmail.com - Writing - Review & Editing

Fernanda Antunes - fernanda_antunes84@hotmail.com – Investigation

Nayara Gusmão Tessarollo - nayaratessarollo@gmail.com – Investigation

Felipe Santiago Chambergo Alcalde - fscha@usp.br – Conceptualization, Methodology, Resources, Writing - Review & Editing

Bryan Eric Strauss - bstrauss@usp.br – Methodology, Resources, Writing - Review & Editing

Viviane Abreu Nunes - vanunes@ib.usp.br – Conceptualization, Methodology, Resources, Writing - Original Draft, Acquisition of the financial support for the project leading to this publication

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Figures
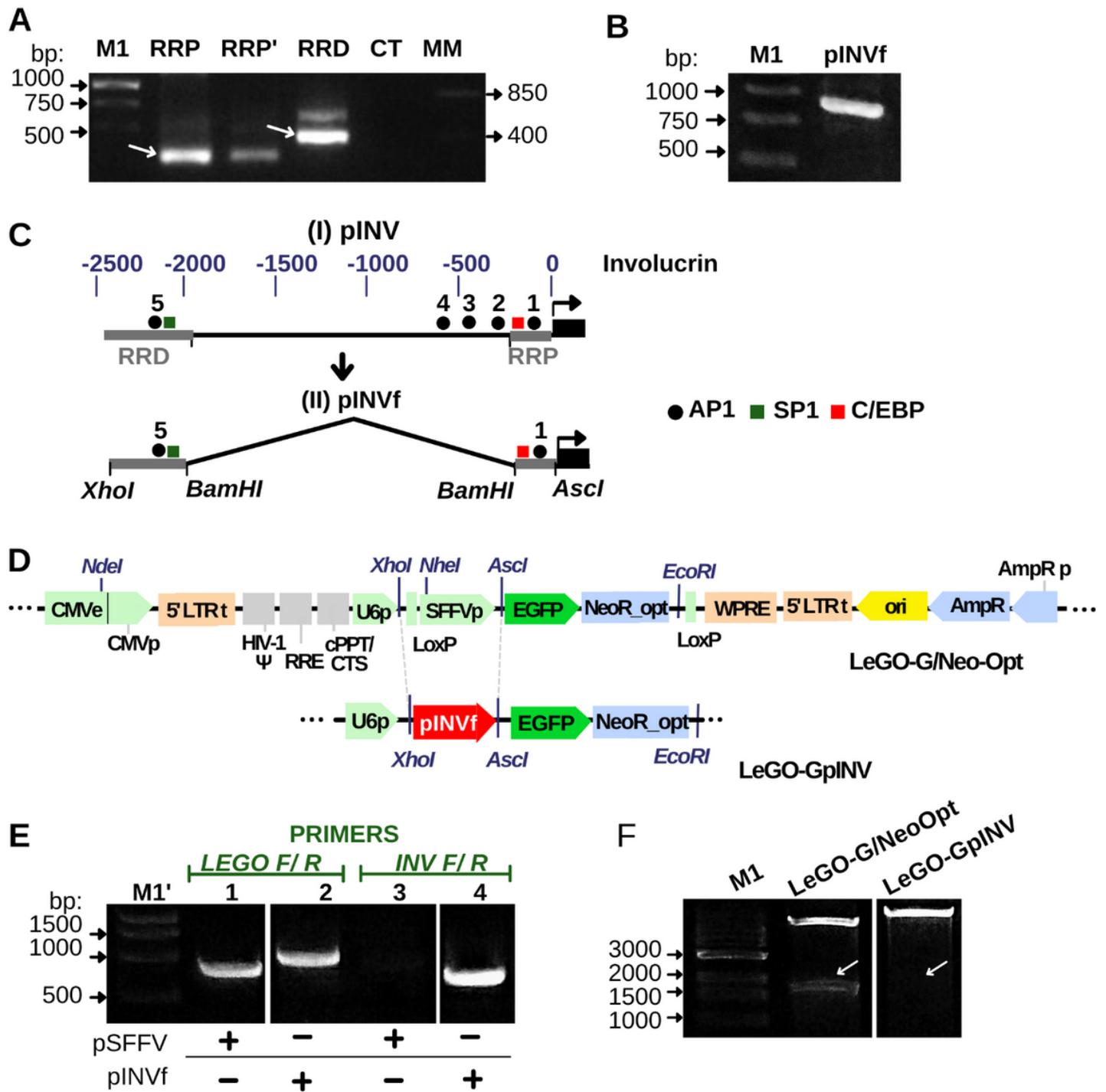
Figure 1

Construction and analysis of LeGO-GpINV. Analysis of products by electrophoresis on 1% agarose gel: (A) PCR products of RRP (242 bp), RRD (404 bp), water (CT); (B) pINVf amplified after ligation of the functional regions forming the segment with 659 bp; (E) PCR products of the vector templates using two primer sets: LEGO-F and R and INV-F and R; the lanes correspond to: 1. pSFFV (726 bp) in LeGO-G/NeoOpt, 2. pINVf (850 bp) in LeGO-GpINV, 3. no pINVf amplification in LeGO-G/NeoOpt, 4. pINVf (659 bp) in LeGO-GpINV; (F) Vector digestion with NheI and EcoRI: LeGO-G/NeoOpt containing pSFFV (1960

bp, arrow), linearized LeGO-GpINV and absence of pSFFV. (C) Scheme of the pINVf construction Adapted from Crish et al. (2006): the pINVf (II) was obtained through the ligation of the RRP and RRD of pINV (I). The sites for AP1-1 and 5, SP1 and C/EBP binding were maintained in pINVf. (D) Scheme of the lentiviral constructions: the pSFFV was replaced by pINVf through digestion in the XhoI and AscI restriction sites. CMVp: Cytomegalovirus promoter and enhancer, 5' LTR t: Truncated 5' Long-terminal Repeat. HIV-1 Ψ: lentiviral packaging signal, RRE: Rev-responsive element; cPPT: central poly-purine tract, U6p: murine U6 pol-III promoter (for shRNA), loxP: recognition sites of Cre recombinase, SFFVp: Spleen focus-forming virus U3 promoter, NeoR_Opt: optimized neomycin resistance, WPRE: woodchuck hepatitis virus post-transcriptional regulatory element. AmpR: Ampicillin resistance. M1: GeneRuler molecular mass standard 1 kb DNA Ladder; M1': Molecular mass standard 1 kb (Amresco Inc, Solon, OH); MM: Fastruler Middle Range DNA Ladder mass standard.
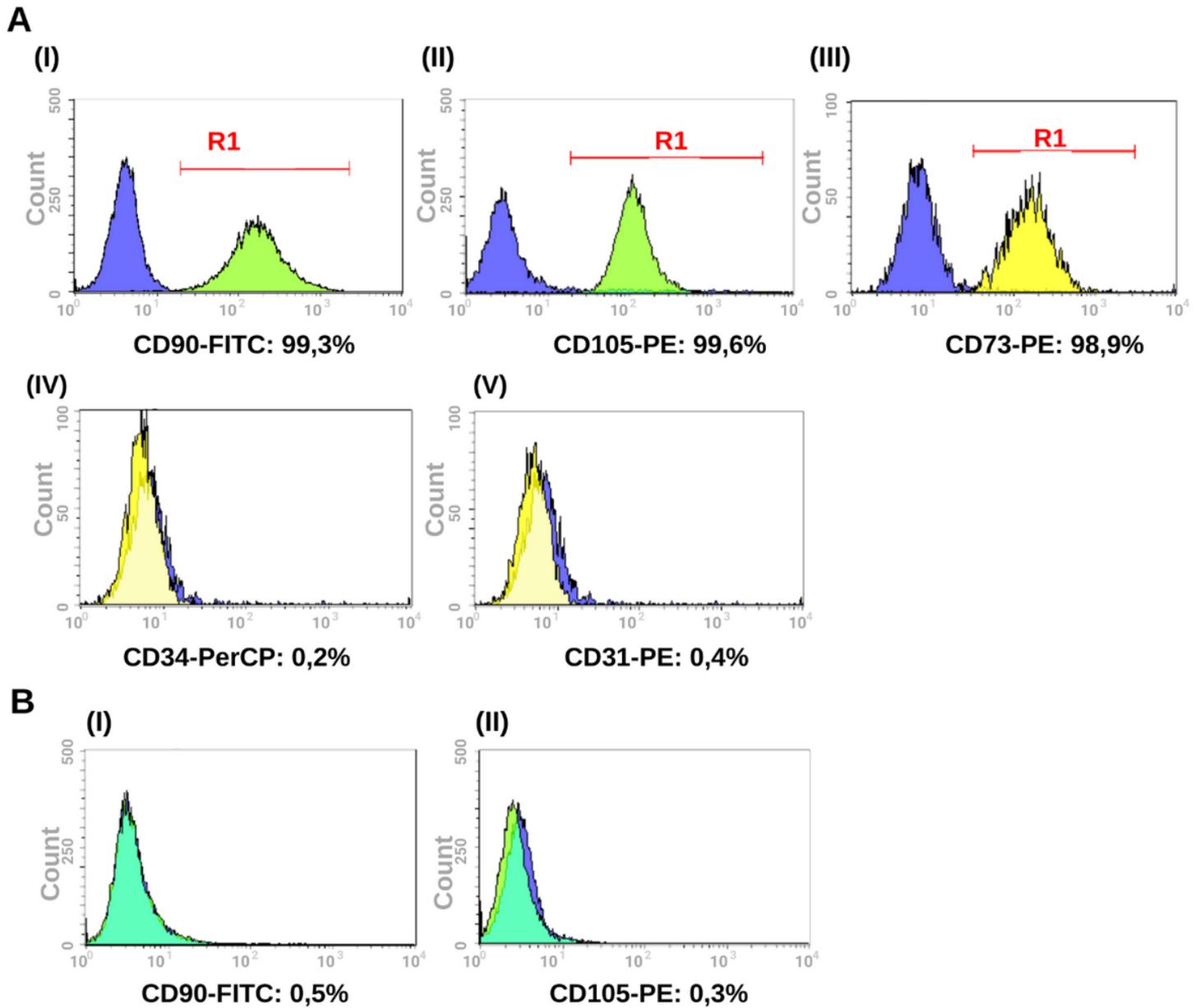
Figure 2

Immunophenotyping of MSC by flow cytometry. Histograms show the identification of surface antigens CD90 and CD105 (green peak) and CD73 (yellow peak) expressed on MSC (A). HaCaT cells (B) were used as negative control for CD90 and CD105 antigens (green peak). The cells were incubated only with the respective secondary antibodies (control, blue peak). The presence of CD90 was analyzed using the filter for FITC detection (525/30 nm), and CD105, CD73, CD31 and CD34 with the filters for PE or PerCP

detection (583/26 nm). Five thousand events were evaluated per sample. The results were expressed in terms of the percentage of labeled cells in relation to the total number of events.
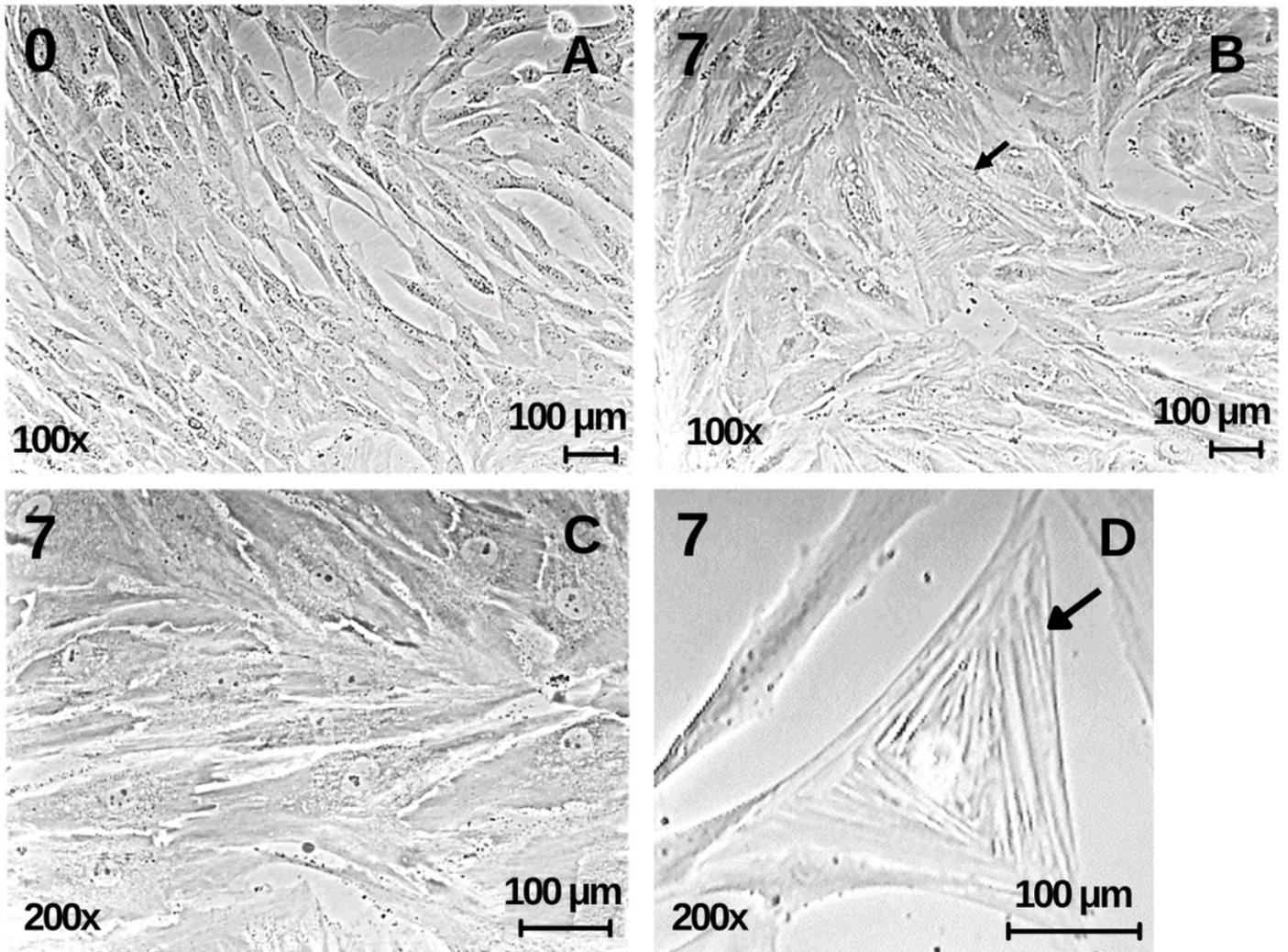
Figure 3

Morphology of MSC induced to differentiate into keratinocytes. Cells grown in proliferation (A) or differentiation medium for 7 days (B to D). (D) The appearance of structures like actin filaments (black arrows). Scale bar: 100 μm. Images obtained under an inverted microscope.
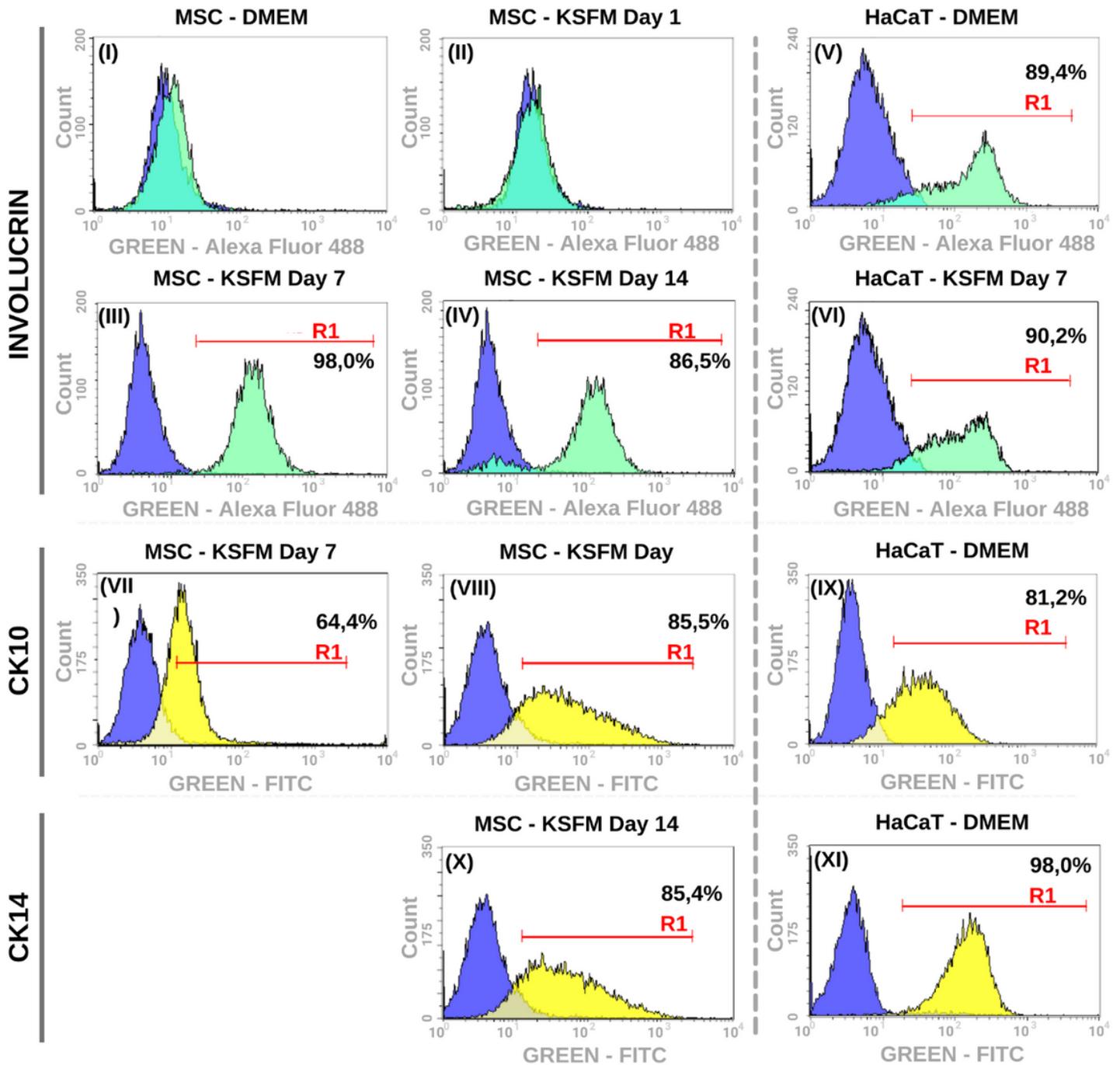
Figure 4

Expression of involucrin, CK10 and CK14 in MSC differentiated into keratinocytes. Histograms showing the expression of involucrin (green peak), CK10 and CK14 (yellow peak). The results were expressed according to the population of cells positively labeled with the anti-involucrin, anti-CK10 or anti-CK14 antibodies in relation to the total number of events analyzed. MSC were induced to differentiate and analyzed in different periods (days 1, 7 and 14) or cultured in DMEM (day 0). Cells incubated only with the secondary anti-mouse IgG antibody were used as controls (blue peak). HaCaT were used as a positive

control for the presence of epidermal markers. Five thousand events were acquired per sample. The cells were analyzed at λ=525/30 nm for Alexa Fluor 488 or FITC detection.
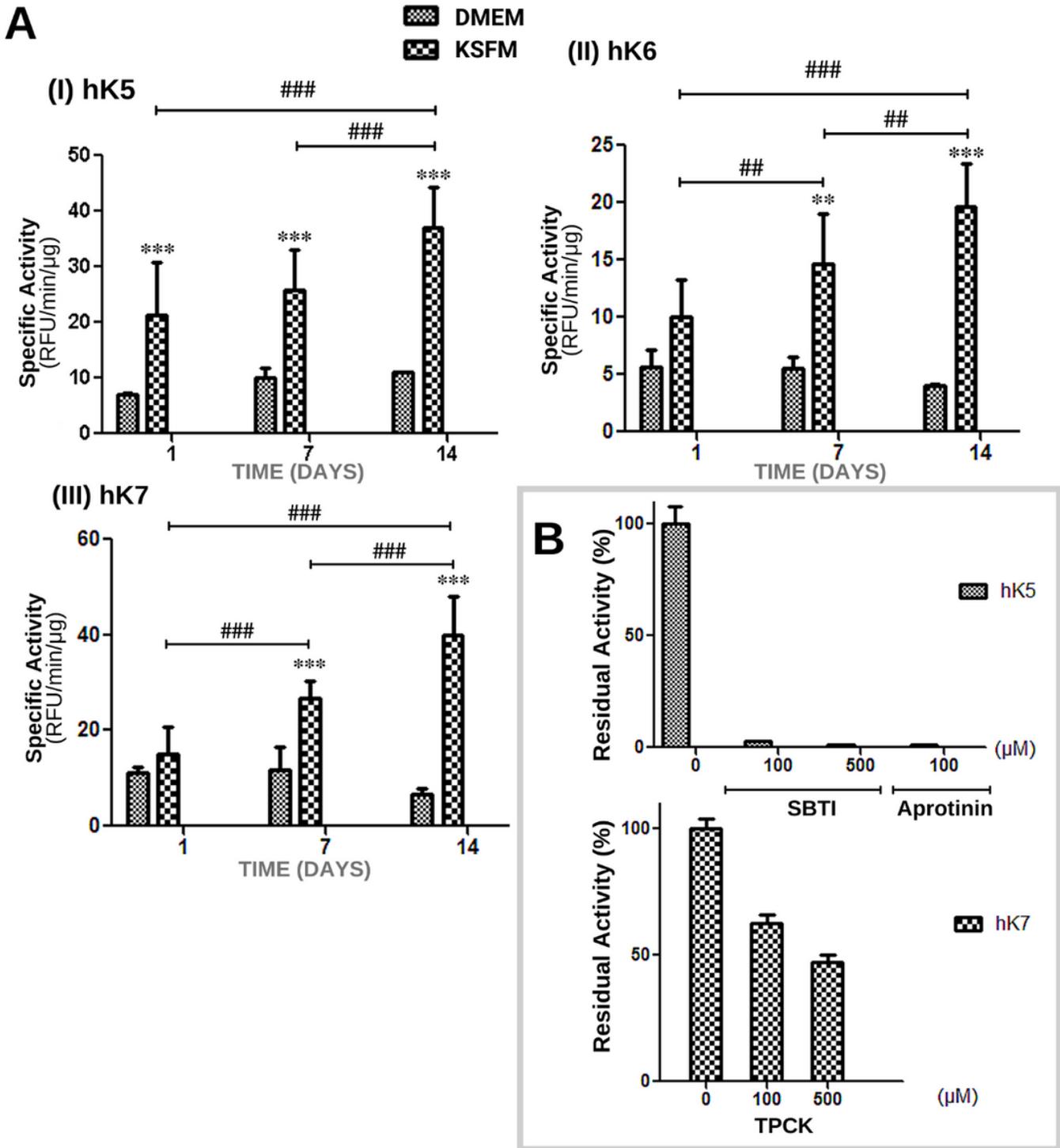

Figure 5

Specific enzymatic activity of tissue kallikreins in the MSC extracts. (A) The specific enzyme activity of hK5 (I), hK6 (II) and hK7 (III) was determined on the hydrolysis of specific FRET substrates. The results were expressed in terms of the average reaction rate per 1 μg of protein present in the extracts. At least four different experiments were carried out in duplicates; (B) The inhibition of enzyme activity in MSC

extracts induced to differentiate. (I) Inhibition of hK5 by SBTI and aprotinin. (II) Inhibition of hK7 by TPCK. The data were expressed in terms of the residual activity of the enzyme in relation to the average rate of the reaction in the absence of the inhibitor.
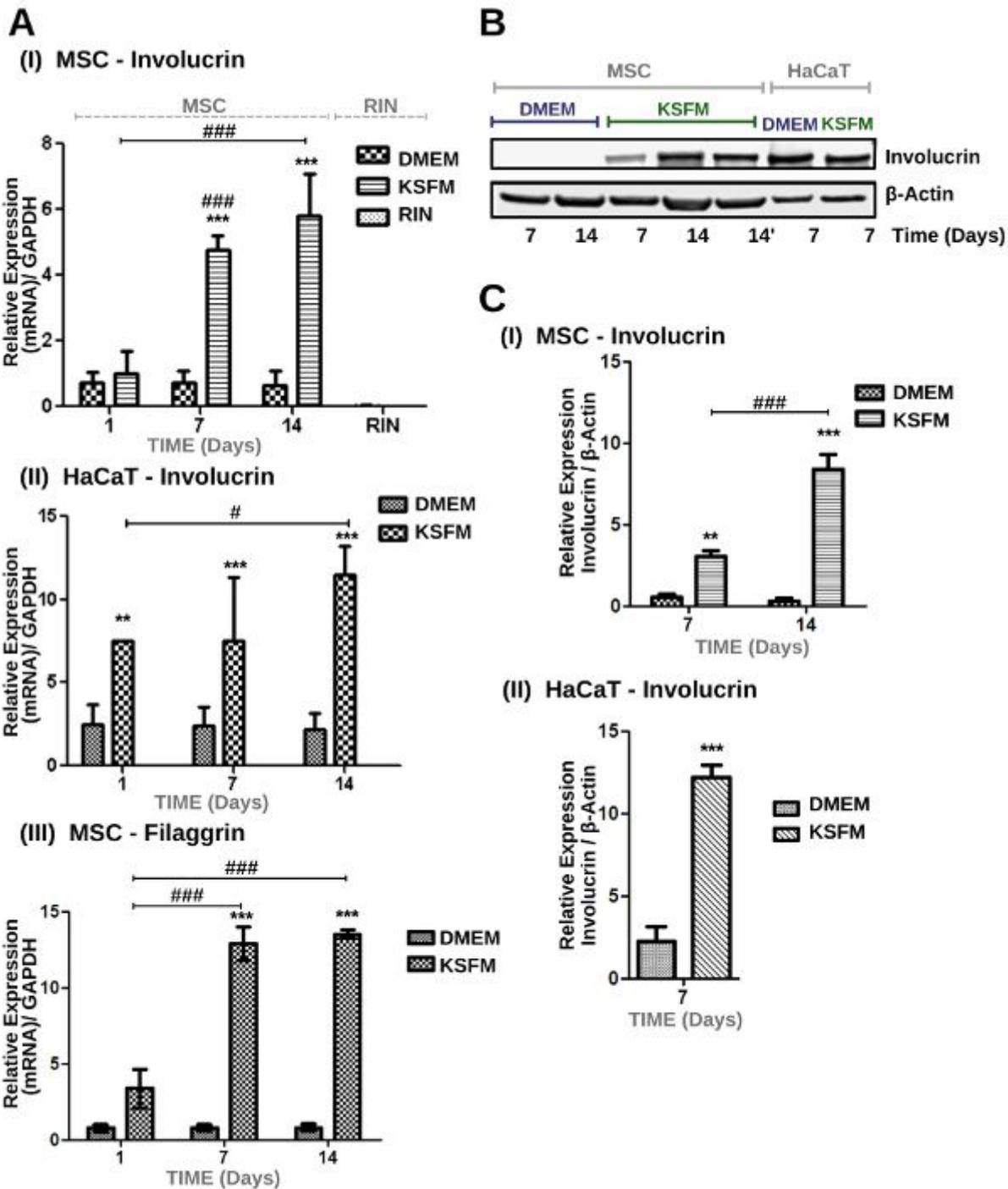

Figure 6

Involucrin and filaggrin expression during epidermal differentiation. (A) Relative expression performed by qPCR of: (I) involucrin in MSC induced to differentiate; (II) involucrin in HaCaT (as positive control); (III) filaggrin in differentiated MSC. RINm5f cells (RIN) and MSC cultured in DMEM were negative controls. The relative expression was determined in relation to the MSC grown in DMEM (negative control) on day

1 and normalized according to the expression of the constitutive gene GAPDH. (B) Relative expression of involucrin by western blot in MSC and HaCaT cultured by 7 and 14 days in DMEM and in KSFM on the same days. 14' corresponds to cell lysates from MSC transduced with the recombinant vector. (C) The densitometric analysis of the relative expression of involucrin was evaluated in (I) differentiated MSC and in (II) HaCaT cells. The results are representative of at least four different experiments, normalized in relation to the expression of β-actin.
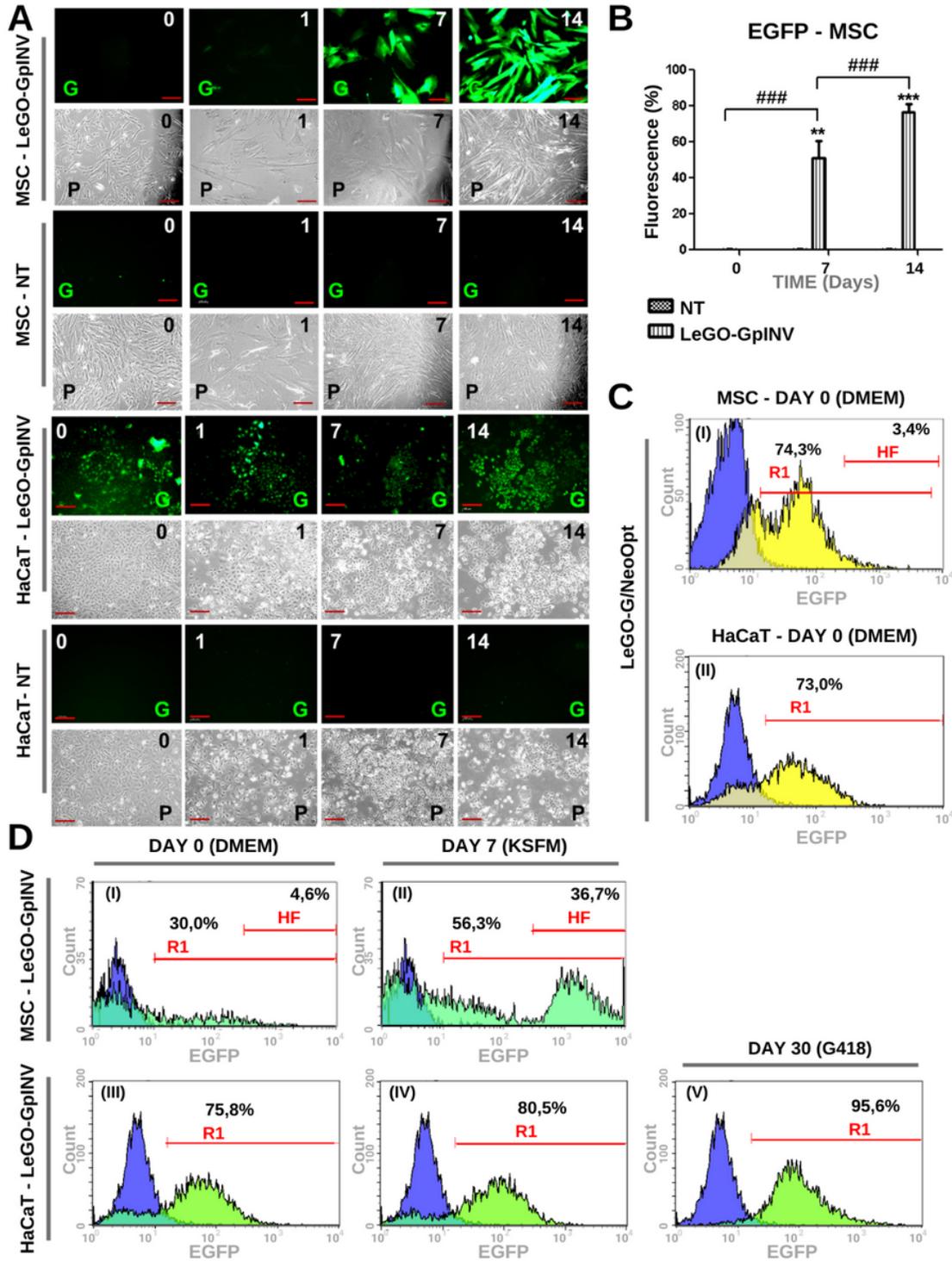

Figure 7

Analysis of EGFP expression in transduced cells. (A) Inverted fluorescence microscopy images of HaCaT cells and MSC transduced with LeGO-GpINV or not (NT) were analyzed on days 0, 1, 7 and 14 of the differentiation periods. Cells expressing EGFP (G) are displayed in green. The images were acquired at λexc=450-490 nm/λem=500-550 nm (EGFP) or in a contrast phase (P). Magnification: 100 x. Scale bar (red line): 100 μm. (B) The mean fluorescence intensity per pixel was compared between MSC transduced with LeGO-GpINV and non-transduced control (NT) on days 0, 7 and 14 of culturing. The mean fluorescence intensity was determined for values above 10 (threshold). (C and D) Representative histograms of the fluorescent cell populations corresponding to the expression of the EGFP reporter protein in MSC and HaCaT (positive control) transduced with the LeGO-G/NeoOpt vector (yellow peak) or LeGO-GpINV (green peak) analyzed by flow cytometry. Non transduced cells were used as control (blue peak). (D-V) HaCaT cells selected with G418 for 30 days. The events were acquired at λ=525/30 nm for green fluorescence detection.

Supplementary Files

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- SupplementaryInfo.MPFernandes.docx