

SHORT COMMUNICATION

Isolating Stem Cells From Skin: Designing a Novel Highly Efficient Non-Enzymatic Approach

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Summary

Stem cells are undifferentiated elements capable to acquire a specific cellular phenotype under the influence of specific stimuli, thus being involved in tissue integrity and maintenance. In the skin tissue self-renewal and wound healing after injury is a complex process, especially in adulthood, due to the aging process and the continuous exposure to damaging agents. The importance of stem cells in regenerative medicine is well known and defining or improving their isolation methods is therefore a primary and crucial step. In the present paper we present a novel method to isolate stem cells from human skin, including the involvement of a novel medium for the maintenance and expansion of *in vitro* cultures. The biopsies were mechanically digested and put in culture. The migrating cells were positive selected with magnetic cell sorting, characterized by flow-cytometry analysis, and viability detected by MTT assay. Cells exhibited a mesenchymal phenotype, as demonstrated by the positive acquirement of an osteogenic or adipogenic phenotype when cultured in specific conditioned media. Taken together our results disclose a novel method for culturing and expanding stem cells from skin and pave the way for future clinical applications in tissue regeneration.

Key words

Stem cells • Cell differentiation • Isolation technique

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Stem cells are undifferentiated elements, able to reply to physical and chemical stimuli from the surrounding environment, by changing cell polarity, differentiation and senescence (Maioli *et al.* 2016). This particular cell-population is engaged in maintaining tissue integrity and replacing damaged elements (Rinaldi *et al.* 2014). Stem cells are located in the so called “stem cell niche”, a protected milieu apparently conserved in different tissue and organisms. The position of stem cells in the niche allow them to remain quiescent until they are enrolled in differentiation or self-renewal processes, according to the specific requirements needed to retain tissue homeostasis (Mao *et al.* 2015, Quan and Fisher 2015). Stem cells can duplicate in two different ways in order to maintain their undifferentiated condition. The first one is a symmetric division which leads to the formation of two stem cells identical to the ‘mother’, the second one is an asymmetric process, in which one cell remain undifferentiated while the other undergo differentiation, with the two resulting cells exhibiting

different features (Khavkin *et al.* 2011). The effectiveness of this process is deeply influenced by cell senescence, slowing down progressively (Maioli *et al.* 2014). An excellent example of this process takes place in the skin during wound healing after injury, a very complex process in which many pathways are activated to obtain a finely tuned response (Gurtner *et al.* 2008).

Skin is the largest organ of the human body and its primary function is to provide a protective barrier for the organism from the environmental injuries. This tissue consists of two different layers: the epidermis and the dermis. The dermis, mainly composed by extra cellular matrix, synthesized by fibroblasts, is located between subcutaneous adipose tissue and epidermis (Quan and Fisher 2015). In the epidermis, the most external part, keratinocytes provide to the formation of stratum corneum full of insoluble bundles of keratine. Indeed, thanks to asymmetric division of stem cells in the basal stratum, mitotically derived cells undergo different fate: one of the cells preserve the undifferentiated status and remains in the basal layer niche; the second one migrates to the upper layers and differentiate, gradually acquiring a dead squames phenotype (Yousef *et al.* 2018).

When the tissue homeostasis is perturbed, extrinsic or intrinsic signals reaches skin stem cells, which may be recruited, becoming epidermal lineages differentiated cells (Mao *et al.* 2015, Quan and Fisher 2015). In adulthood tissue self-renewal and wound healing are difficult processes. Aging affects skin more than any other organs since it is constantly exposed to injuries. Two different processes are involved in cutaneous aging: intrinsic aging, and extrinsic aging. Both of these processes result in a fibrotic tissue that become unable to exert some of the most important function of the skin (Gurtner *et al.* 2008). For these reasons skin stem cells, as well as stem cells coming from other tissues, represent an important resource for regenerative medicine studies (Maioli M. *et al.* 2013), being able to differentiate toward a specific phenotype, under selected stimuli. For example, stem cells isolated from Adipose tissue (Santaniello *et al.* 2018), Wharton jelly (Balzano *et al.* 2019), foetal membrane (Maioli *et al.* 2010) and other tissues have been largely described for their capability to give rise to many cellular lineages, even different from their original belonging tissue. Regenerative medicine is always challenging the discovery of novel safe and easily reliable systems, to implement stem cell regenerative capability.

Stem cells isolation from the skin have been

described several years ago, but still represent a very low yield process obtained after intricate protocols based on mechanical separation and enzymatic digestions, which also significantly affect cell viability (da Silva Jeremias *et al.* 2014, Jeong-Ran *et al.* 2014).

Aim of the present manuscript is to describe a novel non-enzymatic method to isolate stem cells from human skin, by the aid of a specific novel culturing medium suitable for a high rate cell proliferation.

For cell isolation skin biopsies from male and female adult patients (12 samples) from the Dermatological Clinic, Department of Medical, Surgical and Experimental Sciences, in Sassari (Ethical Clearance N. 0021565/2018, 22/03/2018-Commissione Etica CNR) were collected in RPMI1640 (Lonza Bioscience) solution containing 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen) ("Collecting medium" C-medium). Samples were reduced into small fragments of approximately 2 mm x 4 mm in a petri dish by the aid of a sterile scalpels. Fragments of tissue were plated on the bottom of a 35mm culture dishes, three to five pieces for each well with Specific Culture Medium (SC-medium): DMEM supplemented with 15 % of fetal bovine serum (FBS) and 0.1 mM β-mercaptoethanol (Life Technologies, Grand Island, NY, USA), 1 % L-glutamine (Euroclone, Milano, Italy), 1 % nonessential amino acids (Gibco), 200 U/ml penicillin and 0.1 mg/ml streptomycin, FGF (Gibco). Culture were transferred at 37 °C 5 % CO₂ in a humidified atmosphere and medium was changed every 4 days.

One week after the isolation process cell migration could be observed under optical microscope (Leica ICC50 HD). Fragments waste were removed, and cell cultured till confluence, then transferred to T75 flasks (Euroclone, Italy).

The positive selection of stem cells from skin was obtained with magnetic cell sorting using a primary monoclonal anti-c/kit (CD117) antibody (Miltenyi Biotec, Minneapolis, MN, USA) and then cells were magnetically labelled in columns with a secondary antibody directly conjugated to MicroBeads (MACS Miltenyi Biotec, Bologna, Italy). The selected population was then prepared for the flow-cytometry analysis, according to previously described method (Basoli *et al.* 2017).

Cells showed positivity for all mesenchymal surface markers (CD73, CD90, CD105) and were negative for CD31 and CD45 (data not shown). Isolated cells exhibited a mesenchymal phenotype, since were

able to differentiate into osteogenic and adipogenic phenotype (see previously described protocols Basoli *et al.* 2017), (Fig. 1) maintaining this feature also after freezing.

The cell growth curve was measured as indicator of cell proliferation. To define the usefulness of the SC-medium, skin stem cells at passage 1 were grown in this medium and another group of cells were maintained in

a growth basic medium (BM) (DMEM supplemented with 10 % FBS, 200 U/ml penicillin and 0.1 mg/ml streptomycin), considered as control. The number of cells/well was analysed every 24h, using Luna automatic cell counter. MTT assay was performed to test the metabolic activity of isolated cells in SC-Medium and the control BM (Fig. 2).

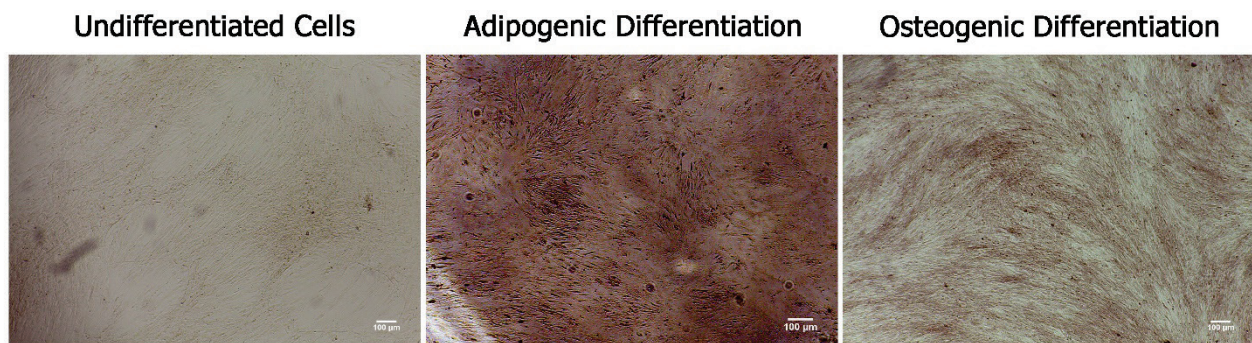


Fig. 1. Differentiation of skin stem cells cultured in a specific adipogenic or osteogenic medium for 21 days. Undifferentiated cells are cultured in basic growing medium. Images are representative of three different experiments. Scale bar=100 µm.

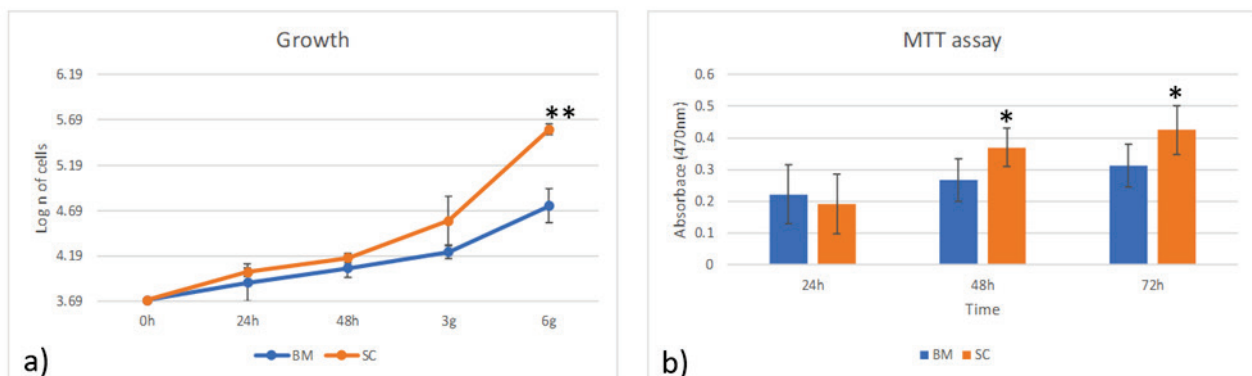


Fig. 2. Growth curve and MTT assay. Statistics are calculated with Anova test (P-value > 0.05)

In the past both stem cells and transit-amplifying keratinocytes were used to regenerate the epidermis, being capable to form the epithelium. Nonetheless stem cells results a more suitable source of elements for skin regeneration, than the keratinocytes progenitor's population (Charruyer and Ghadially 2009). The above described protocol allows a rapid and high-yield isolation of stem cells from skin avoiding the use of enzymatic digestion, significantly affecting stem cell properties and viability (Bianchi *et al.* 2013, Maioli *et al.* 2013). MTT assay and Growth curve show that the presence of SC-medium significantly enhance cell propagation, also increasing their metabolic activity.

Here we describe a novel highly efficient method to isolate stem cells from skin biopsies without

the need of enzymatic digestion. Moreover, we also highlight that this novel medium allows culturing of stem cells preserving their stemness, increasing their viability, and thus optimizing the experimental design. The combination of the SC-medium together with our isolation and culturing method represent a new technique suitable to develop novel strategies for skin regeneration.

Conflict of Interest

There is no conflict of interest

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