Stem cell culture extract (StellActive™) and skin care benefits

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Human mesenchymal stem cells (H™MSCs) are multipotent progenitor cells capable of differentiating into adipocytes, osteoblasts and chondroblasts as well as secreting a vast array of soluble mediators. This potentially makes MSCs important mediators of a variety of therapeutic applications.
Mesenchymal stem cells (MSCs) are potential cellular sources of therapeutic stem cells as their multi-lineage differentiation capacity (e.g., osteogenic, chondrogenic, adipogenic, neurogenic, and myogenic). MSCs can be harvested from various tissues, including adipose tissue and dental pulp, bone marrow derived MSCs (BMSCs).

While all are MSC's, their tissue of residence (or *niche*) has a profound effect on how they function. Those residing in the bone marrow have been proved to be of particular importance in the process of healing. It was recently shown that these cells act as the body's "emergency responders" whenever tissue damage occurs. Injury anywhere in the body can trigger bone marrow MSC's to enter the circulation and then migrate to the place of injury. Once they arrive, some MSC's *engraft* (attach) and *differentiate* (grow into) into specific cell types of the tissue being repaired (e.g. heart muscle in the case of a heart attack). In the early days of stem cell science we thought that this was the primary role of these stem cells – to replace cells that were dead or dying. We were wrong. Their primary effect is to set up a command and control center to coordinate healing and tissue restoration, using other local cells (including tissue-resident stem cells), and immune system cells. This requires the ability for MSC's to communicate with other cells. This chatter between cells uses signaling chemicals called *cytokines*. Individual cytokines, and patterns of cytokines, alter the behavior of local cells -- changing their expression of DNA, production of proteins, metabolism -- all the things needed to lead to healing or regeneration. Cytokines also determine whether a tissue is balanced toward inflammatory healing (fibrosis and scars) or anti-inflammatory healing (fetal-like without scars).

Producing Stem Cytokines in the Laboratory
Cytokines are continuously produced as the cells grow and divide during culture. The cytokine pattern can be modulated using methods that result in preferential production of the cytokines most beneficial in the healing of skin. Lipid encapsulation of conditioned media into nanoliposomes enhances penetration of the cytokines[1].

Of the several cell types mentioned, strong evidence supports the selection mesenchymal stem cells (BM-MSC) as the preferred cell type to obtain cytokines for use in topical anti-aging products for the skin.

MSCs are well known to be capable of differentiating into several cells types including myocytes, chondrocytes, osteocytes, fibroblasts, etc. In fact, MSCs are the predominant cells research focuses on to grow body parts in the laboratory, treat cardiac failure, cardiac infarction, strokes and other medical conditions. Indeed, as part of their role in healing injury some MSCs may differentiate into specific tissue cell types. MSCs participate in the healing process by: 1) controlling and modulating inflammation; 2) stimulating white blood cells to remove debris; 3) triggering division of resident cells to produce more cells; 4) promoting resident cells to produce substances to create intercellular matrix e.g. collagen, elastin, etc.; 5) differentiating into specific kinds of tissue cells needed for repair.

Cell-free lysates prepared from hMSCs were also effective in inducing accelerated wound closure and increased expression of SDF1 and CXCL-5 at the wound bed. Additionally, concentrated media from hMSCs as well as an emulsion containing lysates prepared from hMSCs was also found to be more effective in rapid re-epithelialization than fibroblasts or vehicle-alone control. Use of cell-free derivatives may help replace expensive wound care approaches including use of growth factors, epidermal/dermal substitutes, synthetic membranes, cytokines, and matrix components, and most importantly avoid transmission of pathogens from human and animal products [19].

One goal for skin care is to reduce (or even reverse) the process of aging. So what does the science of wound healing and stem cells have to say about aging? It turns out that skin aging really is a series of poorly healed wounds. Reminiscent of the old saying "death by 1,000 paper cuts", the skin endures damage from UV rays, chemicals and other environmental factors daily. Add to this a diminishing ability to respond with our own natural healing mechanism (MSC's and their cytokine signals) and you see visible progression of the effects of that constant wounding (i.e. aging skin). Regenerative skin therapeutics aims to restore the body's natural capability to be restored to an earlier functional age and appearance. One way to accomplish this is to capture the *essence of healing* (i.e. key cytokines from mesenchymal stem cells) and weave them into a formulation you can apply to skin. This has now been done by many laboratories, and the results have been uniformly positive.

Material and Methods

Isolation and culture propagation of human MSC
Human DMSCs were obtained from dermis of human foreskin. The epidermis was manually removed from tissue piece and incubated in dispase (Gibco) overnight at 4°C, then the dermis was cut into 1-mm3 pieces and incubated in collagenase II (Gibco) for 1 h at 37°C, Dulbecco's modified Eagle's medium with serum free medium, 2ng/mL basic fibroblast growth factor (FGF; PeproTech), 10mM HEPES buffer, 4mM L-glutamine, 100U/mL penicillin, and 100µg/mL streptomycin (all Biochrom). The skin tissue was manually dissociated by pipetting repeatedly and the cell suspension was centrifuged at 1000 rpm for 5 min. The supernatant was removed and the pellet was re-suspended in 10 ml Dulbecco's modified Eagle's medium with serum free medium and 1%

penicillin/streptomycin (Gibco). Afterwards, the cell suspension was transferred to a 25-cm2 non-tissue culture flask. About 2 hrs later, when a small group of cells adhered to the flask, the medium with un-adhered cell was removed. The phenotype profile of DMSCs was confirmed by flow cytometry, as these cells were uniformly positive for CD44, CD73, CD90, CD105 and negative for CD11b, CD19, CD34, CD45.

Human DMSCs were also isolated and cultured by the following procedure: Unprocessed bone marrow (36 × 106 cells/ml) was purchased from Lonza (Walkersville, MD). A Ficoll gradient was used for separation of peripheral blood mononuclear cells (PBMNCs). Isolated PBMNCs were plated in T75 cm2 tissue culture flasks with MesenCult media (Stem Cell Technologies, Vancouver) containing hMSC stimulatory supplements and fetal bovine serum (FBS) for hMSCs. Once cultures were established, several clones were isolated and expanded in culture in the same medium. Established cultures were grown in minimum essential media (α-MEM) containing 10% FBS and penicillin/streptomycin. The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO2. Cells were subcultured every 4 to 5 d and aliquots from passage 2 to 8 were frozen in liquid nitrogen for use. Cell surface markers expressed on these cells were determined by flow cytometry using FITC labeled Abs (BD Biosciences, San Jose, CA) and included Stro1, CD105, CD90, HLA-ABC and CD44 while they were negative for CD45, and CD11b[25].

Several of the passages from 2 to 8 were tested for their ability to differentiate in culture, cytokine production and migration towards keratinocytes and were found to be comparable (data not shown). For the sake of consistency, passage 5 cells were used for all studies reported here.

Cytokine measurement

The cytokine profile of each sample was analyzed using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA) and by Elisa assay for CXCL5 and SDF-1 which was performed on cell-free supernatants from conditioned medium using the Quantikine human ENA-78 ELISA kit (R and D Systems; MN) according to the manufacturer's instructions. All samples were assayed in triplicate. CM was harvested from cultured cells (hMSCs co-cultured with keratinocytes and alone, cultured for 48 h) and filtered through cellulose acetate membrane with 0.45 μm pore size (Corning; NY). hMSCs and keratinocytes were co-cultured (1:5) in serum-free keratinocyte growth medium (Promocell; Germany), cells alone (hMSCs and keratinocytes) were grown as controls.

Statistical analysis

For experiments in this study, individual culture dishes or wells were analyzed separately (no pooling of samples was used). In each experiment, a minimum of three wells/dishes was used and similar results were obtained. Each experiment was repeated a minimum of three times, the mean value of the repetitions was calculated and this value was used in the statistical analysis. Data are presented as mean±SD. The differences were determined

by one-way ANOVA in appropriate experiments followed by Newman–Keuls post hoc test. A probability value of $p<0.05$ was taken to be statistically significant.

Results

Stem cell culture extract (**StellActive™**)

HMSCs were cultured to 60%-70% confluence under standard culture conditions as described earlier. Conditioned medium from hMSCs (5 flasks per experiment with 2 × 107 cells per flask) was collected and further concentrated (50 times) by Amicon ultra centrifugal filter unit with approximately 5 kDa cut-off (Amicon Ultra-15; Millipore, MA) following manufacturer's instructionsAn. This purified stem cell culture extract, **StellActive™**, was tested the purity, the volumetric mass density (ρ), pH. The purified cell medium was filtered by 0.02 μ filters. Aliquot of **StellActive™** was stored under the temperature –20 C. Conditioned medium concentrate (100 μL final concentrate resulting from 5 flasks of hMSC culture medium) from hMSC [hMSC (CMC)] was injected once in the periphery of each wound. Saline (100 μL) was injected in the periphery of wounds as a control and served as the naturally healing group.

Aliquot was also analyzed for cytokine content by multiplex and Elisa as described later.

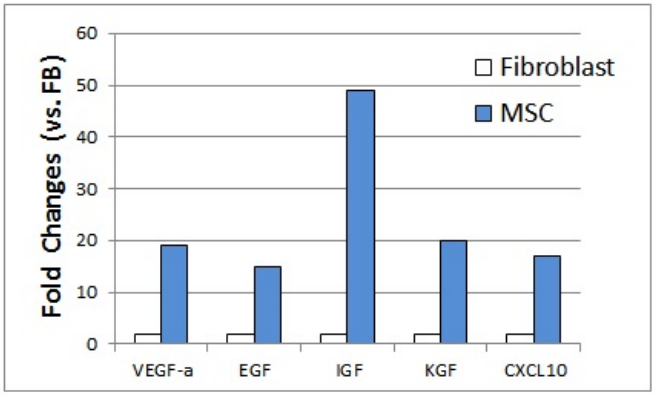
Efficacy studies with **StellActive™**

The focus of these skincare treatments that use stem cells components is to stimulate those stem cells without causing damage, encouraging them to promote the growth of new, healthy skin and collagen to replace old, sun-damaged skin cells, fill in wrinkles and lines and develop a firmer collagen base so skin feels and looks younger and more resilient.
Some stem cell skincare products products derived from them to "communicate" signals to skin stem cells to begin the regeneration process; other products use enzymes or other substances that work in the skin to promote tissue growth.

In vitro study of the efficacy of **StellActive™**

BM-MSC Derived Cytokine Skin Rejuvenation
Topical application of a net anti-inflammatory cytokine admixture produced by BM-MSCs in culture rejuvenates skin.

The accompanying graph below shows the relative production of important cytokines from the two cells types during the culture. (FB refers to fibroblast conditioned media; MSC to bone marrow mesenchymal stem cell conditioned media- **StellActive™**.)



In 2011, Ninety-five women and ten men participated in a multi-month trial of an anti-aging formulation containing BM-MSC derived cytokines (**StellActive™**). Twelve parameters of skin health and appearance were measured including: The results of the trial were highly positive, subjectively and objectively. 65% of subjects considered **StellActive™** the best anti-aging product they had every used. Another 35% considered it better than most products they had tried. Four trial subjects with chronic facial redness and inflammatory condition reported the anti-inflammatory effect of **StellActive™,** confirming the pro-healing anti-inflammatory effect of StellActive™, many users report noticeable benefit on the speed and quality of healing with application of StellActive™ to a variety of injuries. Subsequent **StellActive™** reported benefit following thermal burns, sunburns.

Wound healing in two different mouse models (nude and NOD/SCID)

In the nude mouse model, animals treated with concentrated conditioned medium (**StellActive™)** from cultured hMSCs (CMC) completed wound healing in 6-8 d. Animals allowed to heal naturally took 13-14 d in the nude model.

In the chronic wound model, natural healing takes 24 d. Again, we observed accelerated wound healing when **StellActive™** from hMSCs were administered. Although natural wound healing occurred in both models, **StellActive™** derived from hMSCs clearly accelerated wound healing in both nu/nu and NOD/SCID models.

Discussion

For a number of reasons, bone marrow mesenchymal stem cells appear to be the better source material for cytokine production for topical applications. Whereas liposuction patients are typically well past 40, volunteer marrow donors average 22 years of age. Studies show that cellular function of stem cells declines with age making younger cells preferable. The cytokine pattern of adipose mesenchymal stem cells grown in culture is predominantly pro-inflammatory in contrast to the predominantly ant-inflammatory profile of cells from bone marrow. Inflammation itself is pro-aging, now often referred to as *inflammaging.* Most importantly, the cell population proven to play the primary role in human healing, including skin is bone marrow-derived mesenchymal stem cells. There

are products employing parthenogenetic stem cells (unfertilized embryonic cells) and others that use human fibroblasts instead of stem cells. Some products use a combination of cell types. All have evidence of benefit although good quality clinical trials in humans are hard to find. Based on published research on the science of healing, bone marrow derived mesenchymal stem cells used alone appear most appropriate.

The process of growing stem cells in the lab produces *conditioned medium*, the nutrient broth on which cells feed and where they secrete cytokines as they "talk" to one another coordinating their growth as a cell colony. In products claiming a human stem cell origin, you may find "conditioned medium of …" listed as the active ingredient, often in first position on the label. But not all stem cell types produce the same pattern of cytokines. Stem cells from adipose tissue (fat, discarded from liposuction) have a cytokine pattern in conditioned medium that is considerably different than that of bone marrow derived MSC's.

It is just beginning to understand human stem cell biology and finding ways to exploit this knowledge to reverse visible signs of aging. New cytokines are still being discovered. In the future there may be products with mixtures of cytokines appropriate for different skin types, conditions, and ages. There may be new methods to augment cytokine activity, and new technologies to allow targeting of specific cell populations. Watch for stem cell-derived cytokines employed to improve the outcomes of procedures (surgery, lasers, ultrasound, LED, dermabrasion, dermarolling & others).

Conclusions

In the present study, we demonstrate that **StellActive™** of cell-free derivatives of hMSCs can be successfully used to treat wounds in vivo studies. These studies represent the acute and chronic wound models respectively. Moreover, as they are incapable of mounting an immune response against human cells, wound healing can be studied using **StellActive™**. **StellActive™** stimulates the proliferation of the epidermal cells compensating for the usual deterioration during aging. It leads to an improved epidermal turnover rate and finally to a smoother skin and to a much better skin complexion.

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