Activation of Mesenchymal Progenitor Cells to Correct Signs of Skin Aging

Daniel Schmid, Esther Belser and Fred Zülli
Mibelle Group Biochemistry, 5033 Buchs, Switzerland

Summary
Age-related facial skin sagging is the consequence of reduced activity of connective tissue stem cells. These cells have been identified in the dermal papilla (dermis stem cells) and in the subcutaneous adipose tissue (preadipocytes). An extract of cells of the Argan tree was tested on dermis progenitor cells and found to stimulate their stem cell properties such as sphere formation and expression of Sox2. Tested on preadipocytes, the Argan cell extract enhanced the proliferation of 'aged' preadipocytes. In clinical trials, a cream with the Argan cell extract was found to considerably improve skin density in the papillary dermis and to smooth crow's feet wrinkles.

Introduction
Thinning of the dermis and decrease of the adipose tissue in the hypodermis leads to wrinkles, furrows, and loose, sagging skin. The affected skin areas, dermis and adipose tissue are connective tissues which depend on mesenchymal stem cells for repair and regeneration. Compared with epidermal stem cells, research on dermis stem cells is relatively young, with the first reports about multipotent cells of the dermis appearing in 2001 (1). Experiments on the exact localization of these stem cells revealed that they are located near the hair follicle, in the papilla and the perifollicular area. In 2009, Biernaskie *et al.* showed that dermal papilla cells express the stem cell marker gene Sox2 and that they have a tendency to grow in colonies in the form of spheres (2). These Sox2-positive cells were found to self-renew, to induce the formation of hair follicles and to migrate into the inter-follicular dermis where they proliferated and differentiated to fibroblast cells, able to regenerate the extracellular matrix. The hypodermis is formed by adipose tissue which is important for thermoregulation but has also endocrine functions and it contributes as a natural filler to the youthful appearance of the skin. The hypodermis is also a reservoir of mesenchymal stem cells which are essential for the regeneration of the adipose tissue. These adipocyte stem cells are known as preadipocytes. In order to fight sagging of the skin it is important to preserve the activity of the dermis stem cells and preadipocytes as long as possible. An extract of cells of the Argan tree is shown here to activate dermis stem cells and preadipocytes and thus to redensify the skin and to fill furrows from inside.

Methods
The plant tissue culture technique was used to produce the Argan cell extract. Argan shoots were used to start an argan cell culture line. Callus induction and sub-cultivation was carried out according to standard practice. Incorporation of the dedifferentiated cells in an appropriate liquid media, homogenisation of the cells in suspension and continuous characterisation of the cell suspension was also carried out according to standard procedures. For up-scaling, 10% of the next larger culture volume of a fully grown cell suspension was used as inoculum. Production of biomass was done in 50 to 100 l cultures with a special bioreactor-system (Wave-Biotech AG, Tagelswangen, Switzerland). The extract of argan stem cells was obtained after lysis of the plant cells using high pressure homogenization.
Dermal papilla cells, isolated from excised human hair follicles, were cultured over 6 passages before they were used for primary sphere formation in hanging drops. 16 days after injection of 3000 cells into 10 µl drops, the primary spheres were prepared for immunohistochemical analysis of expression of the stem cell marker Sox2. The cell nuclei were shown by DAPI staining. For the formation of secondary spheres, primary spheres in hanging drops were digested with TrypLE for 30 minutes at 35°C. The cells were counted and seeded at 10'000 cells per well into 24well plates. After 3 weeks, the number of secondary spheres formed was determined.
Preadipocytes obtained from an abdominal plastic surgery on a woman of 61 were cultured in the presence or not (control condition) of the Argan cell extract for one week with a treatment renewal after 3 days of culture. Then the cells were harvested and seeded again in order to obtain 'aged' preadipocytes using the replicative aging model (Hayflick phenomenon). Three series of cultures were performed to obtain cells of passage 5, 9 or 12 and for each series preadipocytes were cultured in presence or not with the Argan cell extract. The proliferation of preadipocytes of passage 1, 5, 9 or 12 was analysed using an ELISA kit measuring BrdU incorporation.
The clinical study was carried out over 56 days with 21 women aged from 39 to 61 having sun damaged skin. An oil in water emulsion with the Argan cell extract was applied twice per day on the

crow's feet area (wrinkle depth) and the inner side of the forearm (density). The emulsion alone was applied on the other half face and forearm. Wrinkle depth was determined by PRIMOS pico and skin density by ultrasonography with the DermScan C at days 28 and 56.

Results and Discussion

Dermal stem cells were isolated from the dermal papilla of excised human hair follicles. Maintained first as a monolayer culture they were transferred into a hanging drop culture system where they formed 3D spheres (Fig. 1), an important characteristic of progenitor cells. In addition, immunofluorescent labeling of whole mount spheres showed positive staining for Sox2, another proposed dermal stem cell marker. When cells dissociated from primary spheres were seeded back into classical cell culture dishes used for routine monolayer culture, numerous secondary spheres were spontaneously formed (Fig. 1). This indicates that once cells have formed primary spheres, they seem to retain a memory of the 3D progenitor phenotype, and preferentially re-form spheres where normally monolayer cultures would be expected. In order to evaluate ingredients for a stem cell vitalization potential, the intensity and uniformity of Sox2-labelling in primary spheres and the number of secondary spheres formed were used as parameters. An extract of cells of the Argan tree was found to enhance the expression of Sox2 in primary spheres and to stimulate the formation of secondary spheres (Fig. 2). Compared to the control culture, the number was increased by 89%. Overall, the results prove the beneficial effect of the Argan cell extract on the stem cell characteristics of the dermal papilla cells. Activation of preadipocytes was studied in a replicative aging model based on the Hayflick phenomenon. Aging could be demonstrated as a reduction in the proliferation capacity. Compared to the cells of passage 1, there was indeed a linear reduction in proliferation of preadipocytes found in relation to the number of culture passages (Fig. 3). Compared to fresh cells, proliferation of preadipocytes that were cultured over 11 weeks was reduced by 60%. But preadipocytes cultured all the time in presence of the Argan cell extract did not exhibit a restriction in their proliferation capacity. On contrary, compared to the control culture after 11 weeks, proliferation of treated preadipocytes after 11 weeks was 6.7 fold higher (Fig. 4). Thus, the Argan cell extract not only prevents reduction in proliferation capacity related to replicative aging, but strongly stimulates proliferation, especially of later passage preadipocytes.

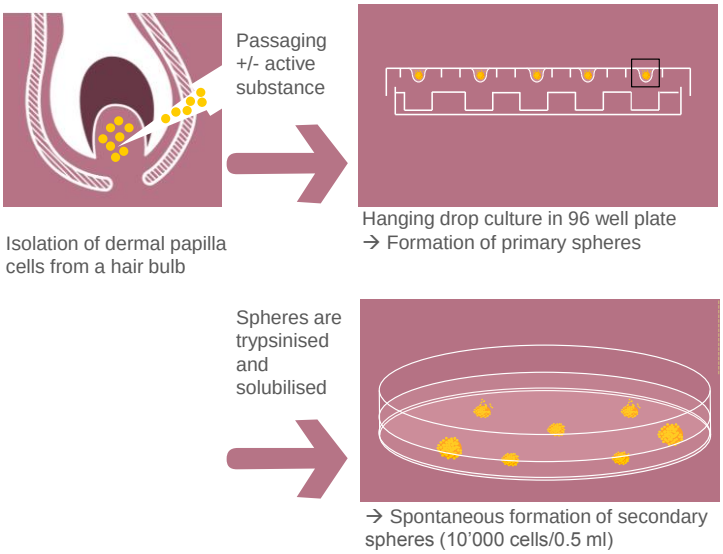

Figure 1: Formation of primary and secondary spheres as test system for dermal stem cell activity

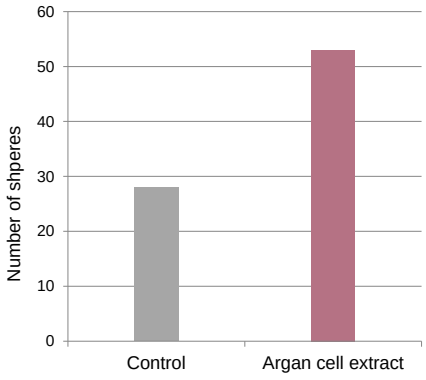

Figure 2: Increased formation of secondary spheres indicate enhanced dermal stem cell activity

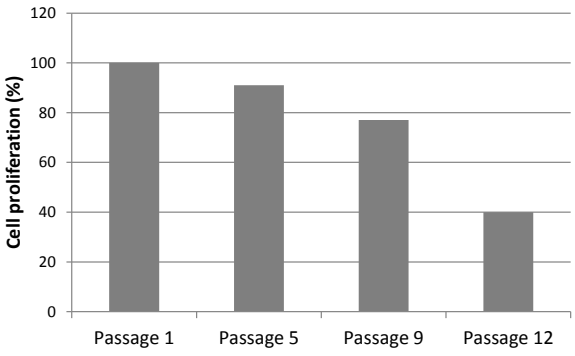

Figure 3: Serial passaged preadipocytes exhibit reduced cell proliferation

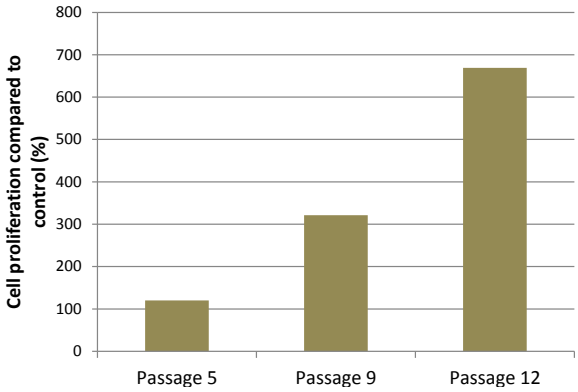

Figure 4: Argan cell extract stimulates proliferation of aged preadipocytes

To test the in vivo effect of the stimulation of dermis stem cells and of preadipocyte activation, an oil in water formulation containing the Argan cell extract was tested on volunteers with sun damaged skin.

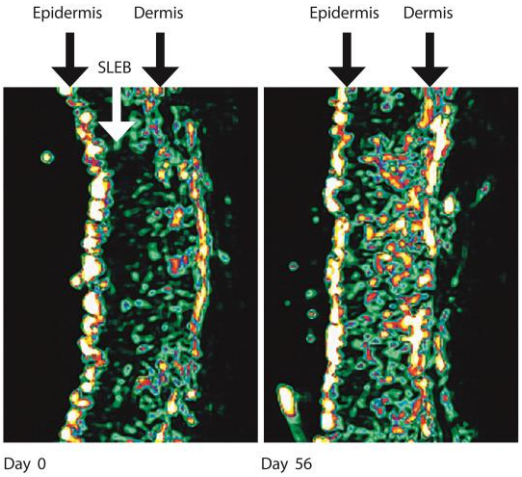

Figure 5: Ultrasonography pictures demonstrate increased skin density

Typical for photo-aged skin is a subepidermal low-echogenic band (SLEB) which is clearly visible in ultrasonography pictures (Fig. 5, left picture). The thickness of the SLEB progressively increases with age and is much more pronounced on sun-exposed skin. Collagen and elastin fibres are degraded in this zone. The results showed that treatment with the Argan cell extract led to a placebo-controlled, significant increase in skin density of 12.7% after 8 weeks. This can be nicely seen on the ultrasonography picture (Fig. 5, right picture). It was also observed that the Argan cell extract successfully reduced the wrinkle depth in the crow's feet area by 26% after 56 days treatment (significant against initial conditions and placebo).

Conclusions

An extract of cells of the Argan tree was found to activate the mesenchymal progenitor cells of the dermis and adipose tissue. Regeneration of these connective tissues depends on active progenitor cells. First results of clinical studies indicate for the Argan cell extract a promising potential to treat facial skin sagging and other signs of skin connective tissue weakening.

References

(1) Toma J.G., Akhavan M., Fernandes K.J., Barnab-Heider F., Sadikot A., Kaplan D.R., Miller F.D. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol*, 3 (9), 778-784 (2001).

(2) Biernaskie J., Paris M., Morozova O., Fagan B.M., Marra M., Pevny L., Miller F.D. SKPs derive from hair follicle precursors and exhibit properties of adult dermal stem cells. *Cell Stem Cell*, 5 (6), 610-623 (2009).