# A novel culture medium ages keratinocytes without the need for pro-aging stimuli

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## Background

A cell's environment plays a dominant role in determining its behaviour, both in vivo and in vitro. The aging process in vivo is an excellent example of environmental changes altering cell function. Age-related loss of cell function is the result of a complex cascade of events (summarised in Fig. 1). The cascade begins with intrinsic and extrinsic aging stimuli including **UV, ROS, xenobiotics, damaged proteins and accumulating metabolites,** that over time are able to overcome the cell's protective mechanisms. These stimuli cause a range of age-related cellular changes including **mitochondrial disruption, altered signaling, DNA damage, apoptosis and senescence**. These changes result in reduced cell function (in particular of stem/progenitor cells), that leads to a reduction in organ function and regenerative potential [1, 2].

The environment is equally critical in vitro, where the ability of specific factors to retain stem cells or drive differentiation is now well known. Culture media also contain a wide variety of highly protective and stimulatory factors to increase growth rate and longevity. As a result, **standard culture media are strongly anti-aging**.

The anti-aging nature of standard media means that traditional methods to induce aging in vitro typically depend on either extended in vitro culture (replicative aging), or exposure to highly concentrated pro-aging stimuli such as peroxide or methylglyoxal. Such methods can induce selected signs of cellular aging, but are either very time consuming, or poorly representative of in vivo aging stimuli.

The new **VitroAge culture medium** (CnT-AG2) uses an alternative approach in which keratinocytes are aged by a culture environment that lacks anti-aging and protective ingredients, without the addition of artificial pro-aging stimuli. Keratinocytes grown in VitroAge medium for 3 weeks were found to retain normal morphology, **but demonstrated reduced growth rate and longevity, disrupted metabolism, and increased protein oxidation.** Proteomic analysis revealed changes in key age-related protein groups: **protein biosynthesis, metabolism, DNA/telomere repair**, **proteosome, basal/progenitor cell markers, and redox/antioxidant mechanisms.**

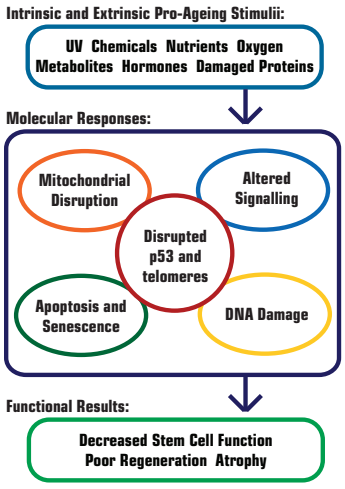



**Figure 1: Schematic of four key molecular responses to pro-aging stimulii [1, 2].**

## Disrupted Metabolism and Membranes

It has recently emerged that the mitochondrial membrane is a key site of age-related changes (1, 2), where oxidative damage to the mitochondrial membrane is and its cytochromes results in increased production of ROS, additional DNA and membrane damage, plus the sustained p53 activation that is now thought to reduce stem cell function and increase apoptosis, senescence, and inflammation.

Keratinocytes grown for 3 weeks in CnT-AG2 medium were found to display a significantly reduced mitochondrial membrane potential (Figure 6). Culture in CnT-AG2 with a protective acitve significantly increased membrane potential (+36% vs CnT-AG2).

Integrity of the cell membrane has long been known to decrease with age, leading to increased permeability [6, 7]. Cell membrane permeability was evaluated by fluorescence in keratinocytes isolated from young and old donors (denoted "in-vivo"), for comparison with young cells cultured in CnT-07 (standard medium) vs cells aged with the CnT-AG2 VitroAge medium ("in-vitro").

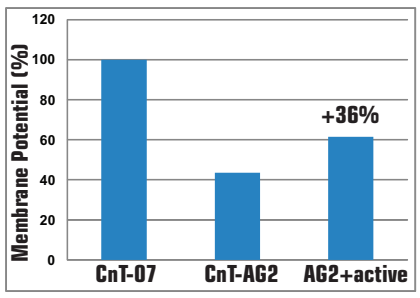
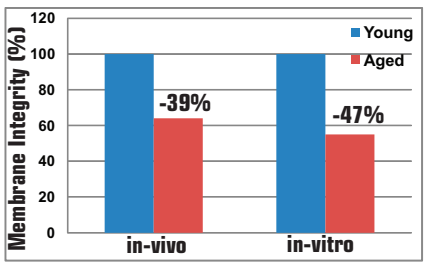



**Figure 6: Mitochondrial membrane potential (JC-1 assay) in primary keratinocytes grown for 3 weeks in either CnT-07 medium, CnT-AG2 VitroAge medium, or CnT-AG2 + a protective active ingredient**.




Membrane permeability was found to decrease in both the in vivo aged cells, as well as the in vitro aged cells (-39% and -47% respectively, Figure 7).

**Figure 7: Cell membrane integrity in primary human keratinocytes. In vivo compares cells isolated from 1 y.o vs 65 y.o donors. In vitro compares keratinocytes growing in standard CnT-07 medium vs CnT-AG2 VitroAge medium.**

## Reduced Proliferation and Longevity

Although epidermal stem cell number appears to remain fairly constant with age, it appears that transient-amplifying cells undergo significant changes, including increased cell cycle duration (up to 3 x longer), and fewer divisions before differentiation [3, 4].

Primary keratinocytes growing in a Progenitor Cell Targeted (PCT) medium from CELLnTEC display many features of transient amplifying prgenitor cells, including morphology and marker expression. In control medium (CnT-07), primary keratinocytes deliver consistent growth rates of 3-4 population doublings per week, typically for 30-40 population doublings.

In contrast, after keratinocytes are transferred to VitroAge medium (CnT-AG2), the proliferation rate declines steadily over three weeks, and then nearly ceases in week 4 (Fig 2). Normal keratinocyte morphology is maintained throughout the ageing process (Fig 3).

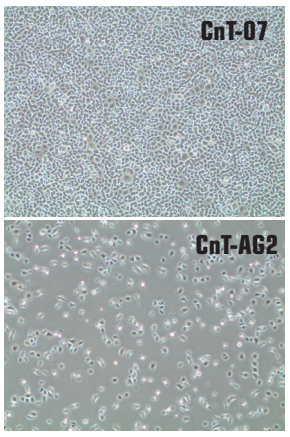



**Figure 3: Keratinocyte morphology in week 3 of culture in standard CnT-07 medium (top), and VitroAge CnT-AG2 medium (bottom). Normal morphology is maintained in CnT-AG2, but proliferation rate is reduced. Day 6 post seeding.**

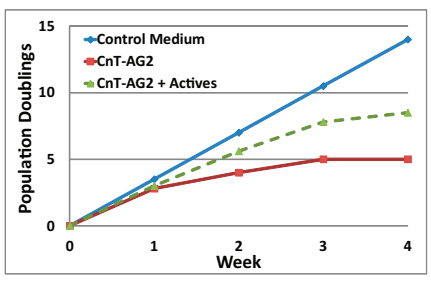



**Figure 2: Cumulative population doublings of keratinocytes in CnT-07 medium (control), and in the 4 weeks following transfer to VitroAge medium (CnT-AG2). Addition of an active ingredient complex resists the aging effect, increasing proliferation vs CnT-AG2 alone.**

## Age-Related Protein Expression

A novel Multiple Reaction Monitoring (MRM) proteomics method developed by Biognosys AG now enables the quantitative determination of up to 100 proteins in a single sample. This method was used to identify changes in keratinocytes after 3 weeks of culture in either CnT-07 (standard) or CnT-AG2 (VitroAge) medium.

Over 300 significantly up- or down-regulated proteins were identified (regulation >20%, p-value <0.05), and grouped into functional clusters using the David Bioinformatic database. Keratinocytes aged in VitroAge medium demonstrated clusters of down regulated proteins associated with protein biosynthesis, metabolism, and DNA/telomere repair. Up regulated functional clusters included proteosome/oxidized-protein-turnover, basal/stem-cell markers, and redox/antioxidant mechanisms.

The MRM analysis of 90 significantly regulated age-associated proteins included the following:

**Metabolism / Protein Synthesis:** glucose-6-phosphate dehydrogenase, ribosomal proteins

**DNA Repair:** Ku70, PARP

**Antioxidant:** peroxiredoxin 4

**Basal / Progenitor Cells:** integrin B4, laminin B3

**Detoxification:** quinone reductase, glutathione-s-transferase

**Stress Response:** 26S proteosome, heat-shock proteins

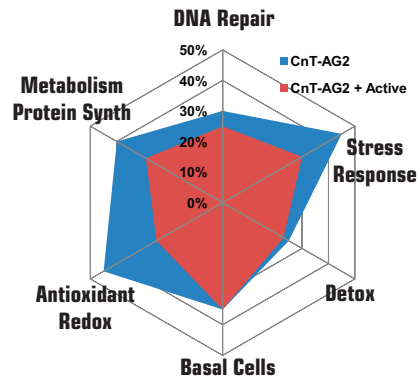



**Figure 8: Percentage changes in expression of age-related proteins in keratinocytes grown in VitroAge medium CnT-AG2 (blue), or CnT-AG2 + protective active ingredient (red). Changes are denoted in percent, vs control (keratinocytes grown in CnT-07). Percentages are absolute values (denote up or down regulation).**

## Increased Protein Oxidation

The accumulation of oxidized proteins (as measured by the protein carbonyl content) in aged tissues is a well documented sign of aging. In many tissues (including skin), protein carbonyl content can double during the second half of an organisms lifespan [5].

FACS analysis of primary keratinocytes grown for 3 weeks in CnT-AG2 (VitroAge) medium (Figure 4) revealed that the level of protein carbonylation increased by 155% compared with cells grown in CnT-07 (standard) culture medium (Figure 5).

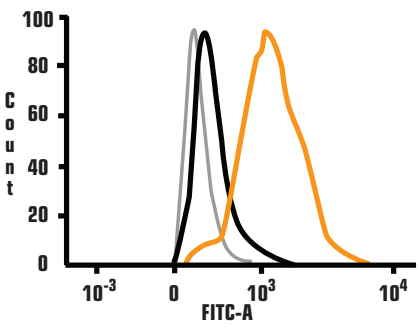



**Figure 4: FACS analysis of protein carbonyl content in primary human keratinocytes grown in either CnT-07 medium (black), or VitroAge CnT-AG2 medium (orange). Cells were treated with DNPH, then probed with an anti-DNP FITC-conjugated antibody. Derivatization control (grey).**

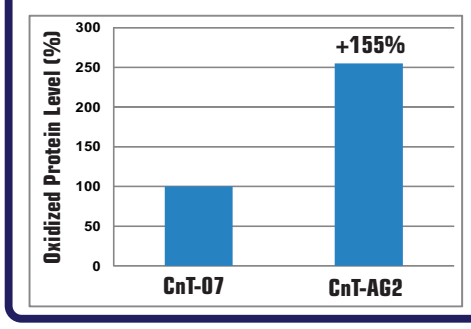



**Figure 5: Level of oxidized (carbonylated) proteins in primary keratinocytes grown for 3 weeks in either CnT-AG2 (VitroAge) medium or CnT-07 (standard) culture medium.**

## Summary and Conclusion

Keratinocytes aged in VitroAge medium (CnT-AG2) for 3 weeks were found to demonstrate a range of changes in cell function and protein expression known to occur with age in vivo. Cells retained normal morphology, but demonstrated reduced cell growth rate and longevity, increased protein oxidation, and disrupted mitochondrial and cell membranes.

MRM proteomic analysis revealed clusters of downregulated proteins associated with protein biosynthesis, metabolism, DNA / telomere repair and electron transport. Up regulated functional clusters included stress response / proteosome, basal / progenitor-cell markers, and redox / antioxidant mechanisms.

These results indicate that keratinocytes grown in CnT-AG2 undergo a variety of phenotypic changes known to associate with age, without the need for synthetic pro-aging treatments such as peroxide or methylglyoxal. Changes in the model can be quantified using a multiplexed MRM proteomics workflow, allowing detailed mechanistic insights into the cellular response to an aging environment, and into the ways in which active ingredients can ameliorate age-related changes.

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