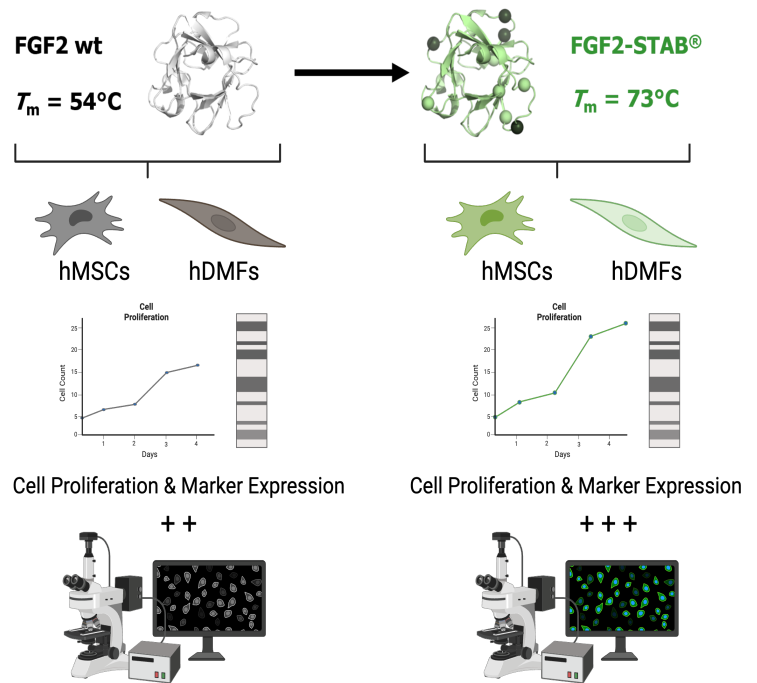
CORE BIOGENESIS

In Collaboration with

Creative Biolabs®

FGF-2 STAB® enhances cell proliferation of MSCs and NHDFs



Background

Fibroblast growth factor-2 (FGF-2) exerts its several functions through the FGF/FGFR interaction. This results in the activation of key molecular pathways promoting the proliferation, maintenance and differentiation of multiple cell types, including mesenchymal stem cells (MSCs). However, most of the studies to date rely on the extrinsic addition of the "wild-type" low molecular weight (LMW - 18kDa) isoform of FGF-2, which is well known for its low stability in cell culture at 37ºC. As a result, the protein starts to degrade rapidly after 10-12hrs, losing its ligand-binding capacity and thus compromising the biological function of those cell types. Given the importance of FGF-2 signalling for triggering the activation of several pathways involved in human physiology, the optimization of culture media formulations to maintain FGF-2 stability and activity over time, is critical for better understanding the biological mechanisms involved in normal development and pathogenesis.

Aim

In this study, we explore how the need for highly pure, active and stable FGF-2 is essential to determine its various downstream effects. Mitogenic activity and FGF receptor-mediated pathways are analyzed, along with marker expression in both human mesenchymal stem cells (hMSCs) and normal human dermal fibroblasts (NHDFs). Comparability analysis demonstrate how the thermostable version of FGF-2, a.k.a recombinant FGF-2 STAB® (Core Biogenesis) outperforms the commonly used "wild-type" FGF-2 on inducing the expression of cell-specific markers and promoting cell proliferation.

Methods

Cell Culture and Proliferation Analysis

Human Mesenchymal Stem Cells (hMSCs) and Normal Human Dermal Fibroblasts (NHDFs) were routinely incubated at 37 °C in a humidified air atmosphere with 5% CO2, and respectively maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with the following conditions: A) No growth factor addition -referred to as negative control; B) Addition of 4ng/mL of "wild-type" human recombinant FGF-2 from Supplier 1 -referred to as positive control/ reference standard; C) Addition of 4ng/mL of "wild-type" human recombinant FGF-2 from Core Biogenesis; D) Addition of 4ng/mL of thermostable FGF-2 STAB® from Core Biogenesis. Passage numbers 1 (hMSCs) and 2 (NHDFs) were used for the experiments. For cell proliferation assays, both hMSCs and NHDFs were seeded in 24-well cell culture plates at a density of 2x10^5 cells/well and incubated in their corresponding media conditions (A,B,C or D). After 7 days in culture without media change, cell proliferation was quantitatively tested using the TC20 Automated Cell Counter (Bio-Rad), by measuring the linear relationship between cell number and signal on a multi-well plate reader.

Western Blot

For immunoblot analysis, cell lysates of hMSCs and NHDFs containing 10μg amounts of total protein were respectively separated by SDS-PAGE and electro-transferred onto membranes.

Membranes were then incubated with monoclonal antibodies to CD73 (for hMSCs marker identity) and to Phospho-Erk (for NHDFs marker of FGF-2-mediated bioactivity). Vinculin was used as loading control for both hMSCs and NHDFs protein contents. Finally, membranes were incubated with secondary antibodies, and detected protein bands were visualized.

Immunocytochemistry

For *in situ* detection, both hMSCs and NHDFs growing on monolayers were cultured for 21 days, fixed with 4% paraformaldehyde, and blocked with 5% bovine serum albumin (BSA). After permeabilization with methanol, cells were incubated with primary antibodies diluted in blocking solution. Primary antibodies were added using the same scheme as in the immunoblotting experiments: CD73 as marker of hMSCs, and Phospho-Erk as marker of FGF-2-mediated bioactivity in NHDFs. Unbound antibody was removed, and cells were incubated with the appropriate secondary antibodies (Thermo Fisher A21206, Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher P36971, ProLong™ Diamond Antifade Mountant with DAPI). Fluorescence microscopy analysis were performed using the Carl Zeiss Axio Observer 5 Inverted Microscope System.

Results

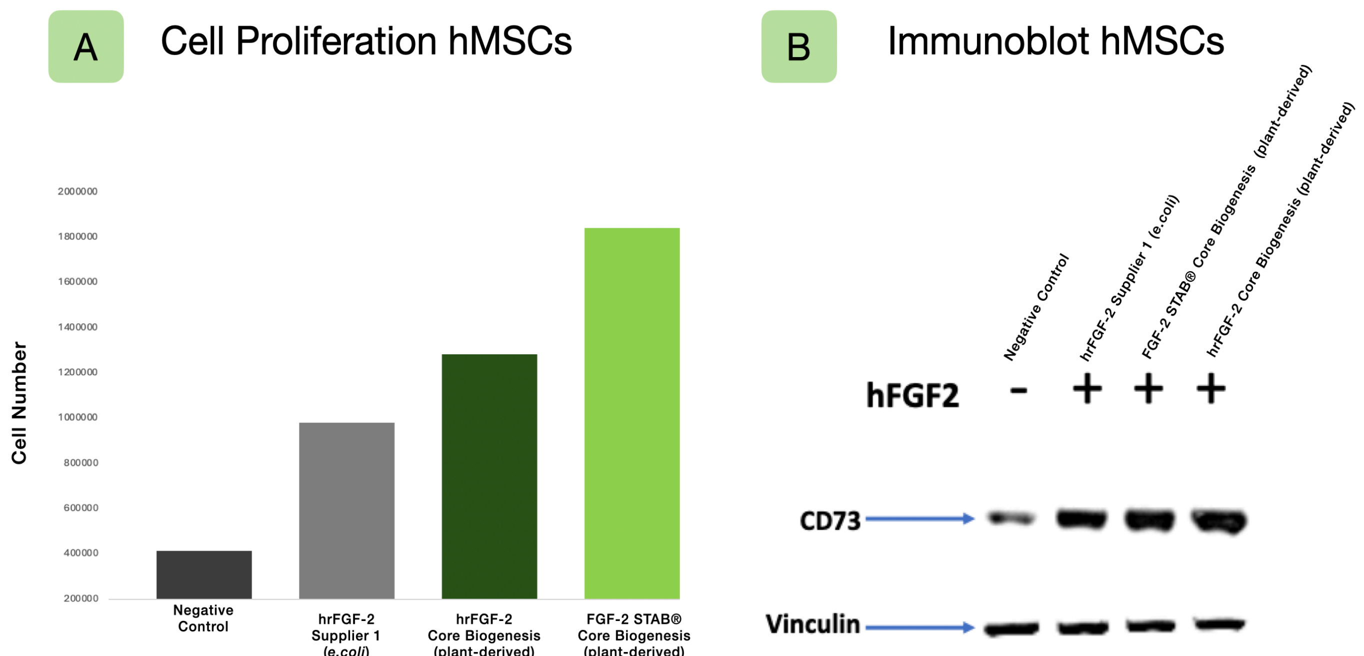
Exogenous supplementation FGF-2 STAB® promotes higher increase of cell proliferation rates than "Wild-Type" FGF-2 on hMSCs

To monitor the correlation between the stable bioactivity of FGF-2 and its effects on the proliferative capacity of hMSCs, cell were cultured under the previously mentioned different media conditions (A, B, C, or D). After 7 days in culture without media change, data shows (Fig.1 A) how the media supplemented with the wild-type version of hrFGF-2 from conditions B (Supplier 1, *e.coli* derived - reference standard), and C (Core Biogenesis, plant-derived) promoted a cell expansion from 2x10^5 (seeding density) to 9.81x10^5 (Supplier 1) and to 12.83x10^5 (Core Biogenesis). Given that both molecules employed in those supplement conditions share the same original sequence, the importance of a highly pure and active product is initially highlighted in the >3x10^5 cell expansion difference. Furthermore, when hMSCs were supplemented with FGF-2 STAB® (Core Biogenesis) cell expanded from the same seeding density to 18.42x10^5 over the course of 7 days in culture. As a result, the thermal stability at 37ºC of this molecule provides a much more consistent activity over time, thus intensifying the process of cell expansion, and allowing hMSCs to achieve optimal proliferation rates.

Stability of FGF-2 signalling correlates with healthy hMSCs morphology and cell marker expression

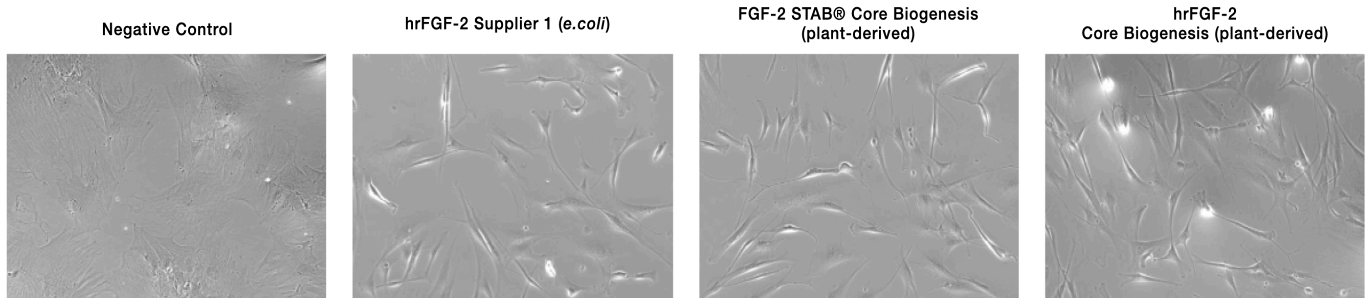
To confirm the identity of the cells and further determine the effects of FGF-2 signalling, hMSCs were characterized by morphology and cell expression markers.

Figure 1



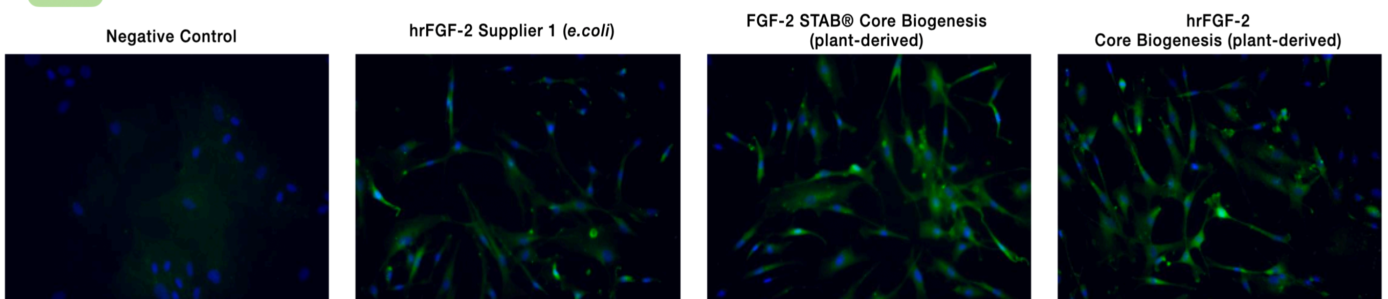
C

Cell Morphology hMSCs



D

Cell Marker Expression hMSCs CD73 (green) DAPI (blue)



As expected, plated cells cultured under all media conditions containing FGF-2 supplement (B, C, D) presented a fibroblast-like morphology specific for MSCs. In contrast, cells cultured in basal media only without FGF-2 supplementation (condition A) started to lose their healthy morphology, and showing a differentiated phenotype. In line with this, FGF-2 is established as an essential factor for preserving the identity and self-renewal capacity of hMSCs. Subsequently, the purity of hMSCs populations was assessed by immunophenotypic profile employing immunoblotting and fluorescent marker expression. The ecto-5′-nucleotidase CD73 is a well-known marker in humans for MSCs. Western Blot analysis revealed CD73 (-) phenotype of cells cultured without FGF-2 supplement (condition A), while all conditions containing FGF-2 in the media formulation (B, C, and D) were CD73+.

Fluorescence microscopy images presented similar patterns. The negative controls established in condition A (no FGF-2 supplement) did not show any CD73 expression as per lack of visualisation of the fluorescent signal. In contrast, cells started to appear CD73+ marked in green as the treatment with FGF-2 supplements were applied. Interestingly, there was a notable difference on the level of immunofluorescence signal of CD73+ between the 3 conditions with FGF-2 supplementation. Cells cultured under wild-type hrFGF-2 from Supplier 1 (*e.coli* derived) presented observable lower signal intensity than with the wild-type hrFGF-2 from Core Biogenesis (plant-derived). This effect was further pronounced when comparing the wild-type hrFGF-2 versions against the thermostable molecule FGF-2 STAB® (Core Biogenesis). Cell viability was also corroborated by DAPI expression (blue) among all conditions.

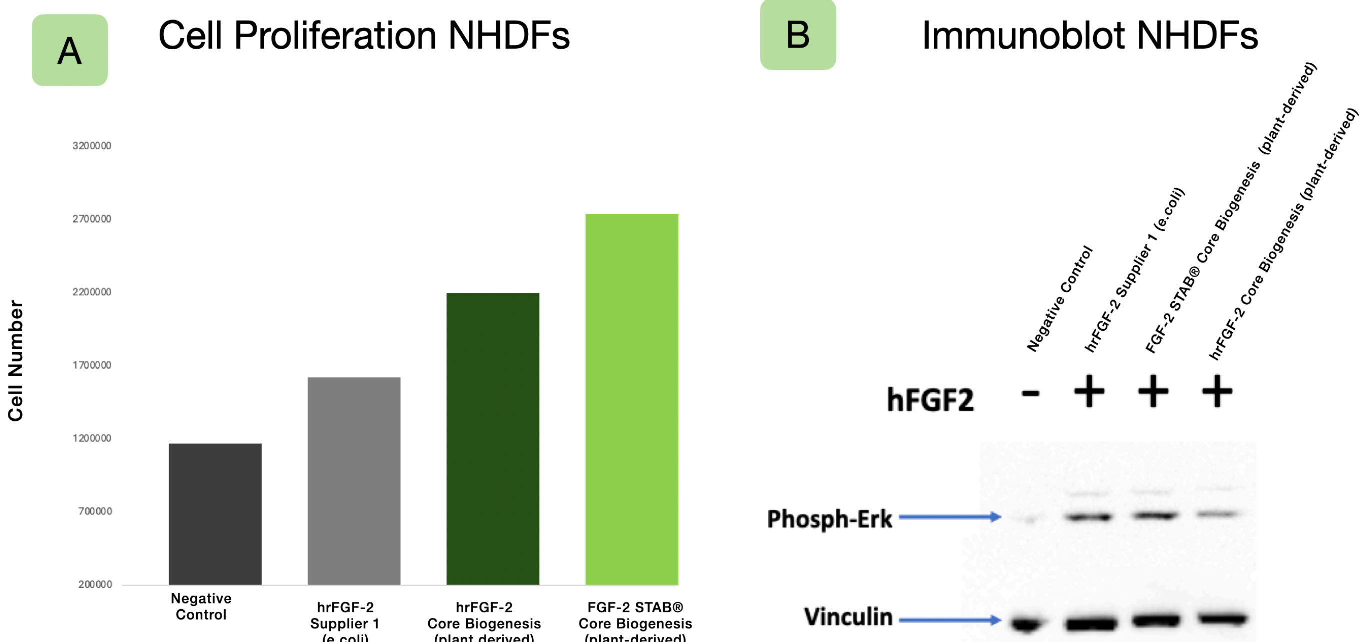
Altogether, these results are in line with the previously shown conclusions on cell proliferation. This data also highlights the effects of FGF-2 signalling, and suggests that the molecule stability on cell culture conditions preserving the ligand-binding capacity and effects through the FGF/FGFR complex, is critical for promoting optimal growth kinetics and preserving the phenotypic properties of hMSCs.

Growth kinetics of NHDMFs are influenced by quality and stability of FGF-2

Cell cultures of normal human dermal fibroblast (NHDMFs) were employed to continue studying the effects of FGF-2 signalling. Literature studies have previously shown the high expression of select FGF receptors (FGFRs) by NHDMFs. This makes them an ideal model to investigate on cell lines beyond those of high interest on stem cell biology (such as MSCs), how FGF-2-mediated extrinsic activity is affected by the quality and stability of this growth factor.

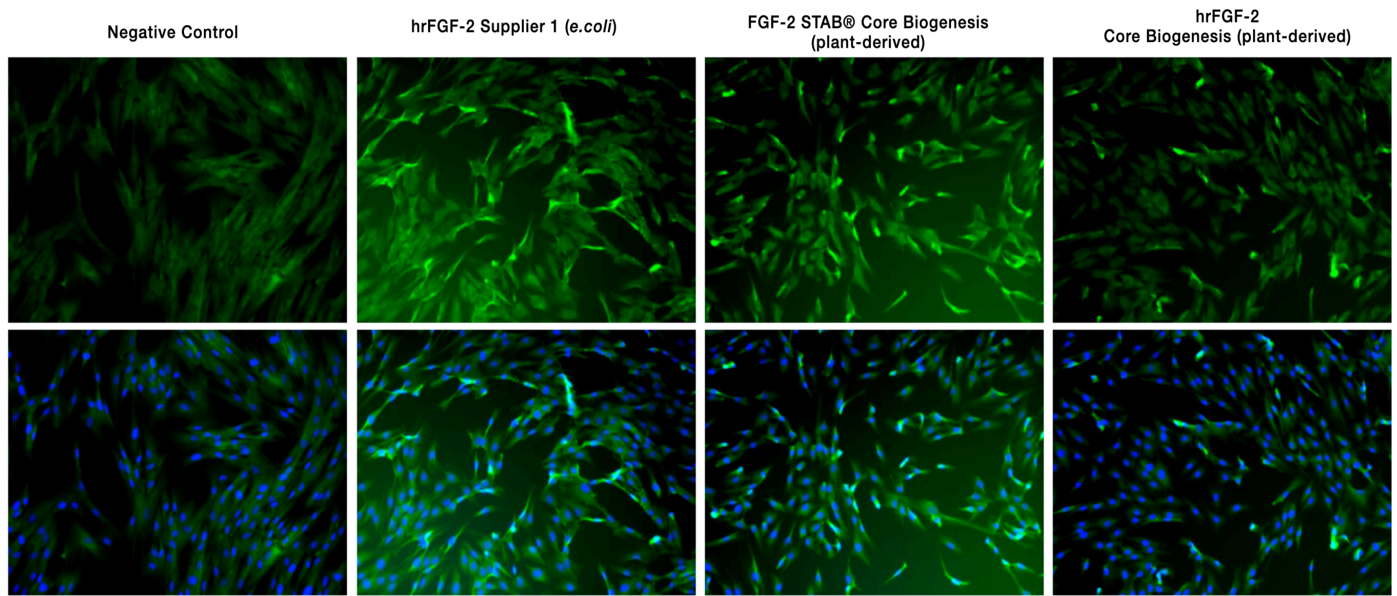
After 7 days in culture and without media change, the growth kinetics of NHDFs differed depending on the FGF-2 version and source. Similarly to the cell expansion analysis performed on hMSCs, the proliferation rate of NHDMFs cultured under "wild-type" hrFGF-2 from condition B (Supplier 1, *e.coli* derived) was significantly lower than on cells cultured on "wild-type" hrFGF-2 from condition C (Core Biogenesis, plant-derived). Starting at a seeding density of 2x10^5 in both cases, the number of NHDFs in culture expanded to 1.62x10^6 and 2.22x10^6 respectively. Optimal proliferation rates were observed after supplementation with FGF-2 STAB® in condition D (Core Biogenesis, plant-derived), promoting NHDFs achieving their maximum expansion at 2.74x10^6 cells. In line with the previous results, the observed >1M cell expansion difference between conditions B to D is a clear indicative of how FGF-2 stability is crucial for achieving high efficiency in cell culture experiments among multiple cell lines.

Figure 2



C

NHDFs Phopho Erk1/2 Expression (green) DAPI (blue)



More importantly, it accentuates how the cell culture quality can be affected if the source of the selected growth factor does not come with high quality and consistent activity.

Activation of the ERK/MAPK pathway increased by FGF-2 STAB®

The biological activity of FGF2 on the different media conditions (A, B, C and D) was further qualitatively assessed by investigating the growth factor supplementation ability to activate the ERK/MAPK pathway. For this, both western blot and immunocytochemistry analysis with phospho-specific antibodies were used to evaluate weather each growth factor ("wild-type" hrFGF-2 from Supplier 1 / "wild-type" hrFGF-2 from Core Biogenesis / and hrFGF-2 STAB® from Core Biogenesis) induced phosphorylation of ERK1/2, and subsequently activated the MAPK pathway.

First, western blot analysis demonstrated all FGF-2 coming from the different supplier sources and stability versions were able to activate the MAPK pathway by phosphorylation of ERK1/2, in a similar manner. Immunocytochemistry confirmed the presence of phosphorylated ERK1/2 in NHDFs cultured for 14 days and treated with all conditions of FGF-2 (B, C, and D). Notably, the fluorescent signal coming from phosphorylated ERK1/2 (green) in NHDFs cultured with FGF-2 STAB® was the strongest among all conditions. These findings put together the biological evidence of how the increased stability of FGF-2 STAB® results in a consistent signalling activation of crucial cellular pathways for cell survival, phenotypic maintenance and expansion, and thus developing higher cell culture qualities for cell biology and stem cell research studies.

Acknowledgments

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