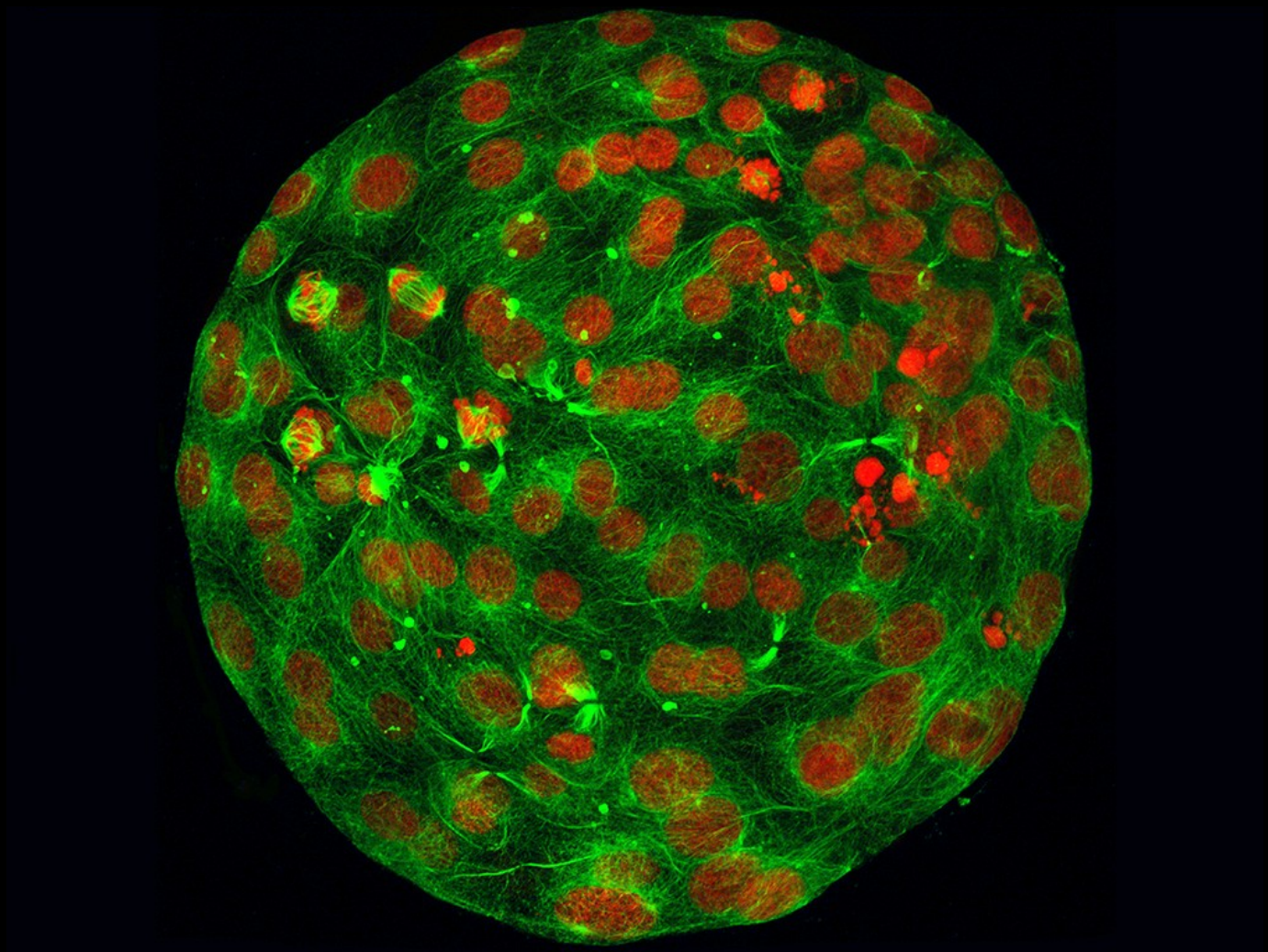
CORE BIOGENESIS



Evaluating the use of Thermostable FGF-2 (FGF-2 STAB®) for optimized iPSC Culture Protocols

Application Note

In collaboration with TreeFrog therapeutics

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Evaluating the use of Thermostable FGF-2 (FGF-2 STAB®) for optimised iPSC Culture Protocols

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Introduction

Human-induced pluripotent stem cells (hiPSCs) have become pivotal in contemporary research, providing a sturdy foundation for intricate disease modelling and the reliable development of innovative therapies. However, challenges arise in their cultivation, notably in technical considerations including the understanding of cell sources, maintenance, quality control, and study design, together with the linked cost implications. One of the biggest obstacles in iPSC culture is the requirement for daily media changes, which is mainly driven by the instability of fibroblast growth factor-2 (FGF-2), a crucial component for maintaining pluripotency. FGF-2 is prone to proteolytic degradation, compromising its effectiveness in regulating biological processes. As a result, the short half-life of FGF-2 (< 10 hours) poses difficulties in sustaining signalling cues for pluripotency, impacting cell health and homogeneity.

Researchers are actively seeking alternatives to overcome these barriers and enhance the efficiency and cost-effectiveness of iPSC culture techniques. In this study, we evaluate the performance of the thermostable version of FGF-2, referred as FGF-2 STAB® for maintaining high quality iPSC cultures, while allowing stem cell researchers to integrate optimized cell feeding schedules. This protein tackles the FGF-2 decay with an enhanced half-life (> 7 days at 37°C), developed by computer-assisted engineering, and enabling to create a new variant with 9-point amino acid substitutions that preserves the full bioactivity and signalling required for FGF-2 mediated pathways. Furthermore, the added benefits of this growth factor are empirically evaluated by direct testing in standardized iPSC methods, including the assessment of cell morphology, expansion capacity, and pluripotency markers expression. Sourced from Core Biogenesis, FGF-2 STAB® comes as the only existing plant-derived version, which enables for an endotoxin-free expression system and produces an animal-free, tag-free protein. These distinctive advantages provide a stable, and cost-effective alternative for iPSCs culture, while supporting the scientific community to embrace more sustainable practices and materials, thanks to the environmentally responsible manufacturing of recombinant proteins in plant systems. Altogether, the results of this study demonstrate how FGF-2 STAB® can be adopted as the new gold-standard solution for advanced stem cell research, and the future of cell-based medicines.

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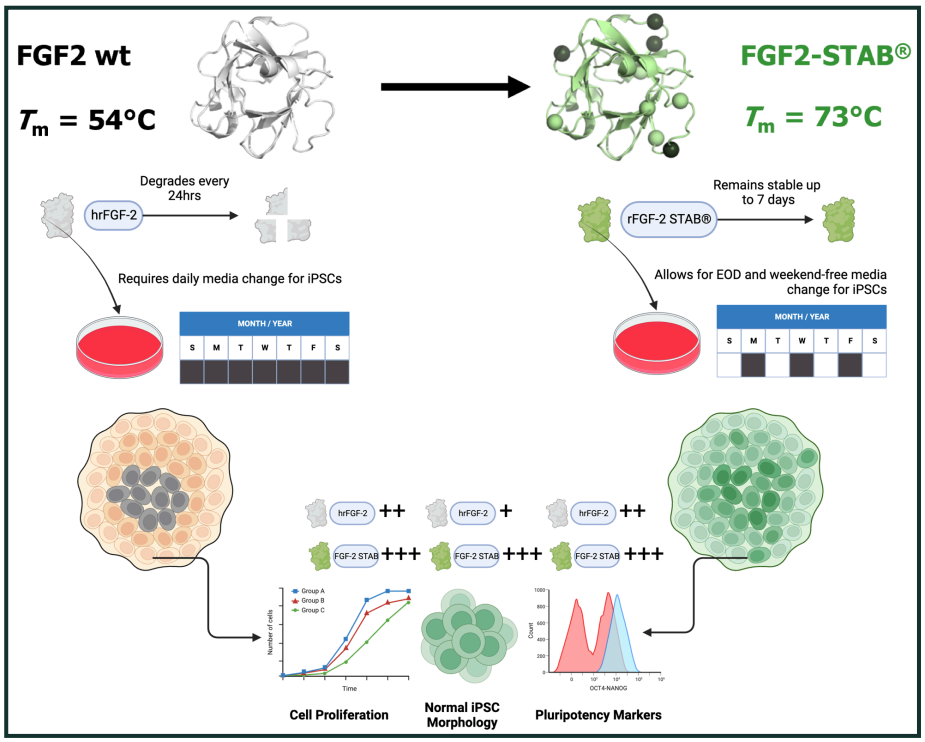
Graphical Abstract



Highlights

- **FGF-2 STAB® allows for superior quality iPSCs cultures**, promoting **high cell proliferation rates**, while **maintaining a stable pluripotent** phenotype.
- **Consistent signaling levels of FGF-2 STAB®** permits for same feeding schedules defined on **weekend-free iPSC culture** protocols, and commercial media kits.
- Core Biogenesis **FGF-2 STAB®** provides significant **cost savings** for iPSC research and manufacturing projects.
- Plant-derived **FGF-2 STAB® from Core Biogenesis contributes to reducing the environmental impacts** coming from stem cell research and manufacturing activities.

Statement of benefit

Over 3,000 publications are published every year containing the topic of iPSCs. The major applications are developmental biology, disease modelling and drug screening. In addition, there are over one hundred clinical trials exploring the therapeutic potential of iPSCs. This study aims to support the advancement of the fields of stem cell research and therapy by addressing important challenges including: The development of new materials that allow for optimized cell performance; cell culture strategies that reduce the environmental impacts of the culture media, while improving quality, cost-effectiveness and downstream scalability.

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Materials and Methods

Cell Culture

iPSCs were cultured in Laminin 521 (2.5µg/mL) coated 6-well plates. Cells were grown in 3 different media conditions: A) StemFit Basic 03 (Ajinomoto) + 50-100 ng/mL human recombinant FGF-2 (Core Biogenesis) B) StemFit Basic 03 (Ajinomoto) + 50-100 ng/mL recombinant FGF-2 STAB® (Core Biogenesis) C) mTeSR Plus (Stemcell technologies). During the experiments, cells were passaged 2-3 times a week, every 3 or 4 days depending on cell confluency. After passaging, cells were reseeded in media A, B or C containing 10µM of ROCK inhibitor (Y-27632) during the first 24 hours. Later, media was changed to either A, B or C conditions without ROCK inhibitor. To evaluate the performance of FGF-2 STAB® against conventional Human FGF-2, and commercial media kits containing thermostabilized FGF-2, media changes were performed as follows: Daily for media conditions A (StemFit + Human recombinant FGF-2) and B (StemFit + recombinant FGF-2 STAB®). Every other day for media conditions B (StemFit + recombinant FGF-2 STAB®) and C (mTeSR Plus).

Growth Factors for iPSC Culture

The capacity of FGF-2 STAB® to optimize iPSC culture was evaluated by direct comparison with its native analogue, Human recombinant FGF-2. Both Human recombinant FGF-2 and recombinant FGF-2 STAB® were obtained from Core Biogenesis, and consist of animal-free, tag-free growth factors with high bioactivity, and produced under sustainable manufacturing practices. FGF-2 STAB® (Core Biogenesis) contains the 154aa mature domain of FGF-2, with 9aa point mutations, resulting in an improved thermal stability of the molecule, without impacting its bioactivity. As a result, the active half-life of this growth factor is increased from <10hrs (for Human recombinant FGF-2) to >7days (for recombinant FGF-2 STAB®), when employed in cell culture media at 37ºC. This patented molecule was originally developed and described in the work of Devorak et.al 2018. Core Biogenesis was granted the license from Enantis, to manufacture, release, and distribute recombinant FGF-2 STAB®, resulting in the most cost-effective and sustainable product on the market, thanks to the proprietary plant-based bioproduction.

Pluripotency Markers analysis by Flow Cytometry

iPSCs were rinsed 2 times with PBS and incubated in media (A,B,C) with Accutase for 3 minutes at 37ºC. After cell detachment, cells were collected and centrifuged for 5 minutes at 300g. Cell pellets were resuspended and cell counting was performed by following the manufacturer's instructions for NucleoCounter® NC-200. Later, cells were passed through a 40µm cell strainer until obtaining a single-cell suspension. iPSCs were fixed and permeabilized using the eBioscience diluent (ThermoFisher). Following cell fixation and permeabilization, cells were incubated with primary antibodies: Nanog-APC, OCT4-PE, SSEA4-Viogreen, SSEA5-Vioblue. 1,000,000 events were collected using MACSQuant Analyzer 10. Cell viability was determined by flow cytometry analysis, and data on pluripotency marker expression was analysed using FlowJo software.

Results

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iPSCs Supplemented with FGF-2 STAB® Display Normal Pluripotent Morphological Phenotypes

As a first step, the quality of iPSC cultures under different media conditions was evaluated by morphological appearance under the microscope. More importantly, this method was also applied to observe any spontaneous differentiation, thus indicating non-optimal cell culture conditions to preserve the pluripotent profile of iPSCs. When cells were cultured only with basal media StemFit Basic without growth factor supplementation (Fig. 1A), cells presented an epithelial-like differentiated morphology. In line with the standards for pluripotent stem cell culture, the use of basal media only lacks the appropriate extrinsic signalling molecules required to maintain stemness, including the control of the FGF-2/ERK pathway.

These results correlate with the morphological observations presenting non-pluripotent cells, and is also associated with spontaneous differentiation. As expected, cells cultured under growth factor supplementation (Figure. 1B-F) displayed healthy colony forming morphologies typical of pluripotent stem cell phenotypes. To investigate the effects of the stability of FGF-2, iPSCs were cultured with StemFit + FGF-2 STAB® (Core Biogenesis) at 50 ng/mL or 100 ng/mL following 2 different feeding regimes during 5 days in culture: I) Cell feeding and media replacement with additional FGF-2 STAB® every day (Fig. 1B-C); II) Cell feeding and media replacement with additional FGF-2 STAB® every other day -EOD-, as shown (Fig.1 D-E). Microscopy images were acquired at day 4, showing how cells under both feeding schedules presented similar morphologies, with no spontaneous differentiation observed.

Figure 1

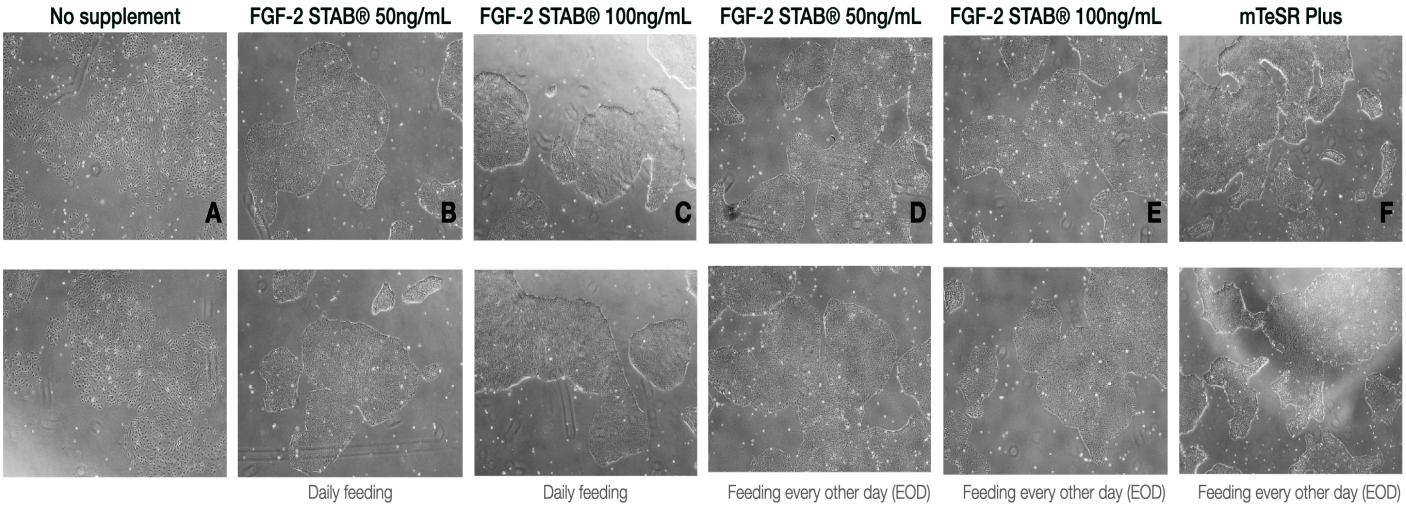

Fig.1 Microscopy imaging of iPSC morphology and viability analysis. iPSCs were cultured in StemFit Basic, with no growth factor supplements (A), StemFit Basic + 50ng/mL FGF-2 STAB® with daily feeding (B), StemFit Basic + 100ng/mL FGF-2 STAB® with daily feeding (C), StemFit Basic + 50ng/mL FGF-2 STAB® with feeding EOD (D), StemFit Basic + 100ng/mL FGF-2 STAB® with feeding EOD (E), mTeSR Plus with feeding EOD (F). Cells were monitored daily and images were taken at Day 4 after passaging and subsequent media feeding under described conditions. Representative 4X images (2 per condition) acquired with EVOS M5000 system.

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Interestingly, even the lower concentration of 50ng/mL was able to achieve this effect, suggesting both the potency and sustained protein stability of this growth factor also at lower doses. The performance of FGF-2 STAB® for maintaining high quality pluripotent stem cell cultures was also evaluated by comparing its effects against mTeSR Plus, which also contains stabilized FGF-2 in its composition. Culturing iPSCs in this commercial media with improved formulation and designed for weekend-free feeding, provided similar cell growth and morphological appearance to FGF-2 STAB® supplementation. Notably, daily monitoring of cell growth under the microscope showed slightly different colony sizes and cell growth rates, suggesting that cells cultured under mTeSR Plus achieved confluency sooner. Further analysis will be needed to define the genetic stability of these effects. Additionally, no-direct correlation of the differences between the FGF-2 STAB concentration used to supplement StemFit, and the one from mTeSR Plus can be established, as the concentration of FGF-2 stabilized in mTeSR Plus is not disclosed.

Cell Proliferation Rates of iPSCs change upon Media Composition, and Growth Factor Concentration

The growth rates of iPSCs under different concentrations of both “wild-type” human recombinant FGF-2 and recombinant FGF-2 STAB® (Core Biogenesis), were assessed by cell proliferation assays. Viable cells and average amplification data from 3 different passages indicated that both growth factor variants exert similar effects on cell proliferation at concentrations of 50 ng/mL and 100 ng/mL when supplemented into StemFit Basic, and if new media and growth factors are added every day. After 5 days in culture following this scheme, the lower concentration (50 ng/mL) promoted an average amplification rate of 73% for human recombinant FGF-2, while for recombinant FGF-2 STAB® the amplification rate corresponded to 83%. The effects of adding a higher concentration of 100 ng/mL resulted in an amplification rate of 83% and 85%

Figure 2

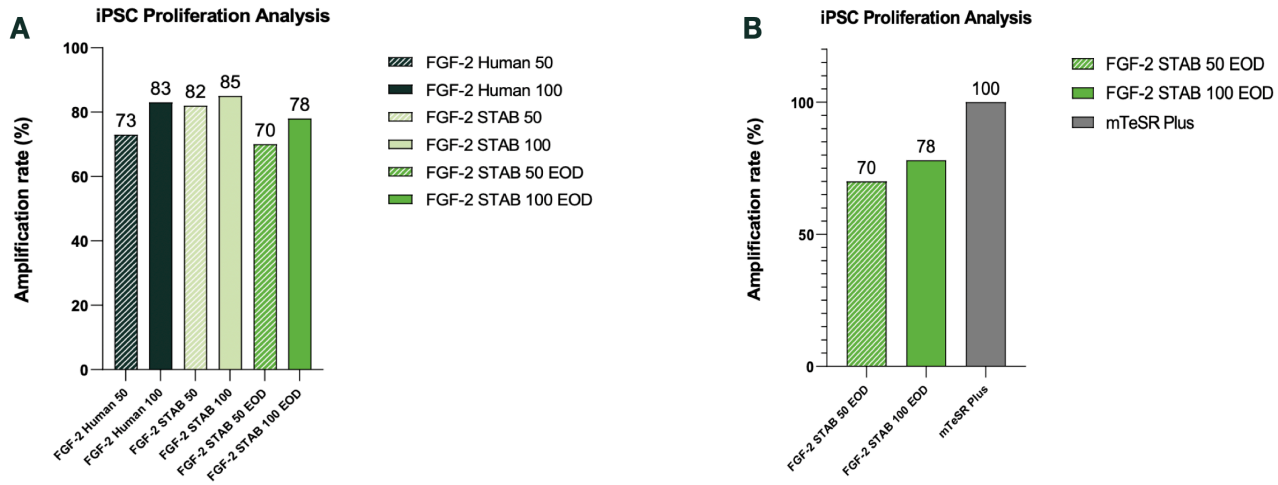

Fig.2 Bar charts showing the average amplification rates of (**A**) iPSCs cultured in StemFit supplemented with Human Recombinant FGF-2 (Core Biogenesis), or Recombinant FGF-2 STAB® (Core Biogenesis) at concentrations 50ng/mL and 100ng/mL during 5 days, following feeding protocols of media change every day (for both Human Recombinant FGF-2 and FGF2 STAB®), and media change every other day (EOD) for FGF-2 STAB®, and maintaining the indicated concentrations. (**B**) Comparison of average amplification rates of iPSCs and confluency, when cultured for 5 days with media change EOD, between formulations based on StemFit + FGF-2 STAB® (at 50ng/mL and 100ng/mL), and mTeSR Plus. Each mean is based on 3 independent measurements.

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for human recombinant FGF-2, and FGF-2 STAB® respectively. As expected, these results suggest that the bioactivity of both human recombinant FGF-2 and recombinant FGF-2 STAB® is kept similarly when the growth factors are added daily. The 2-10% difference with an increased amplification rate for FGF-2 STAB® demonstrates the improved potency of this molecule. It also correlates to the better stability of the protein, as the degradation of human FGF-2 ("wild-type) is already observed every 24 hours. Moreover, the concentration of 100 ng/mL for both types of FGF-2 promoted the optimal growth of iPSCs.

The capacity to maintain the signaling levels of FGF-2 STAB® at 37ºC thanks to its thermal stability, was also demonstrated when studying the cell proliferation of iPSCs under every other day (EOD) media change strategy . For this case, FGF-2 STAB® promoted the iPSCs numbers be amplified by 70% at 50 ng/mL, and 78% at 100 ng/mL, being consistent with the previous concentration dependent findings. Compared to the daily media change, the cell proliferation was slightly lower in EOD. This result suggested that after 48 hours, the amount of growth factor available in the cell media is lower than when added every 24 hours. However, the protein quantity decay due to cell consumption, is counteracted by the presence of stable growth factor in the media, as it is still able to promote cell proliferation at high rates.

Furthermore, the proliferation kinetics of iPSCs stimulated by FGF-2 STAB® supplemented in the StemFit Basic media and added EOD, was compared to the amplification achieved by mTeSR Plus under the same feeding conditions.

With 5 days in culture, iPSCs reached confluency with mTeSR Plus and achieved maximum amplification rate (established at 100%). For FGF-2 STAB®, the rates were preserved as previously at 70% for 50 ng/mL and 78% for 100 ng/mL. All results were obtained from experiments repeated in triplicates, and cell viability was > 90% for all conditions.

Based on this data, it can be established that Core Biogenesis FGF-2 STAB® can achieve optimal growth and maintenance of iPSCs at 100 ng/mL, preserving its function with reduced weekly feeding schedules, and alleviating the demanding culture conditions for pluripotent stem cells. At the same time, the results comparison with commercial complete media formulations such as mTeSR Plus, suggests that FGF-2 is the most essential growth factor, for which its stability remains crucial to preserve pluripotency. Moreover, other components present in these commercial media, normally based on published formulations such as E8, support to maximize the cell growth, while keeping all the essential external signaling pathways needed to maintain pluripotent stem cell phenotypes.

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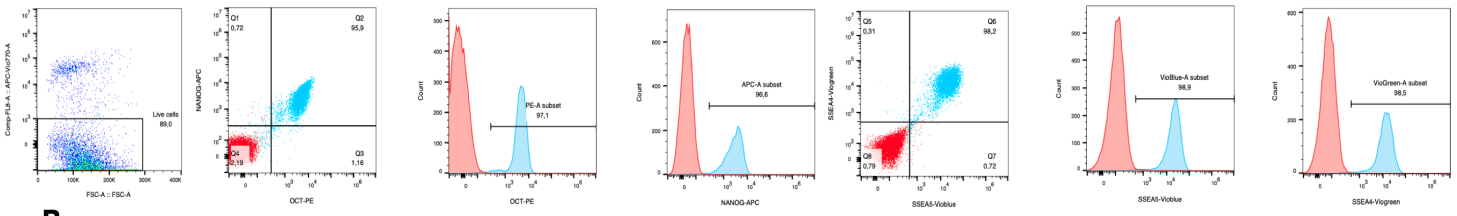
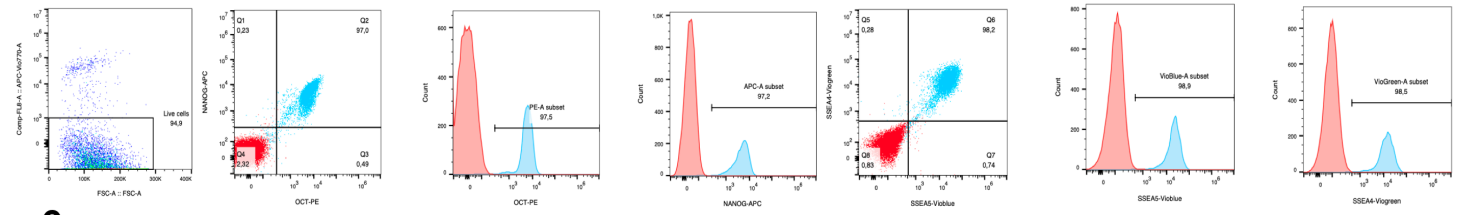
Figure 3

iPSCs Culture Conditions

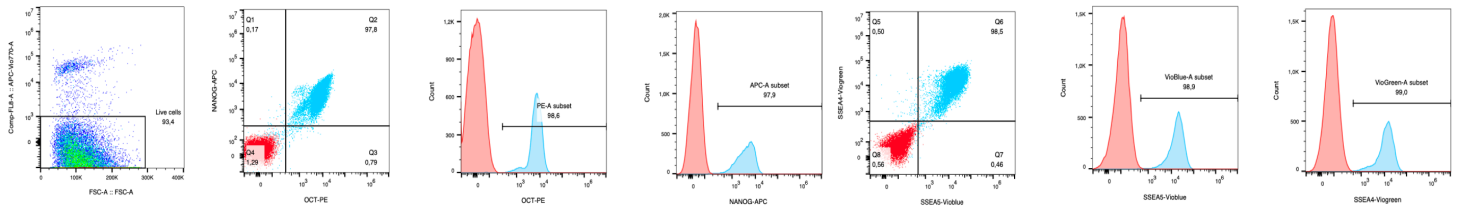
A Human Recombinant FGF-2 50 ng/mL Every Day Feeding



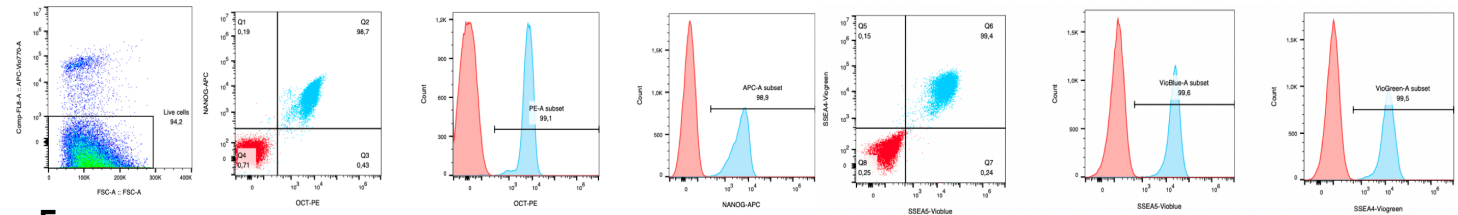
B Human Recombinant FGF-2 100 ng/mL Every Day Feeding



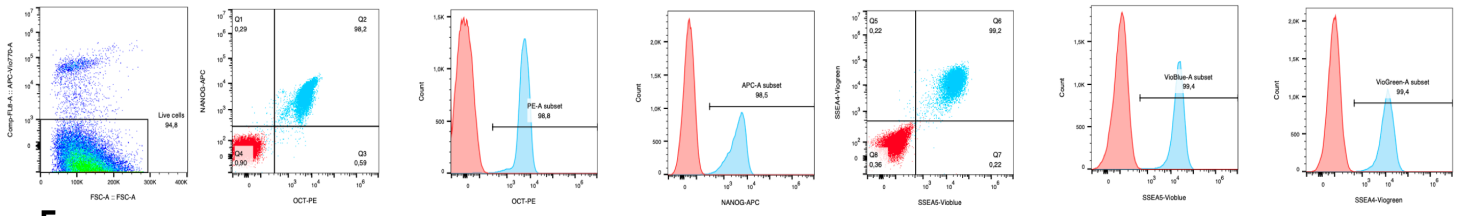
C Recombinant FGF-2 STAB® 50 ng/mL Every Day Feeding



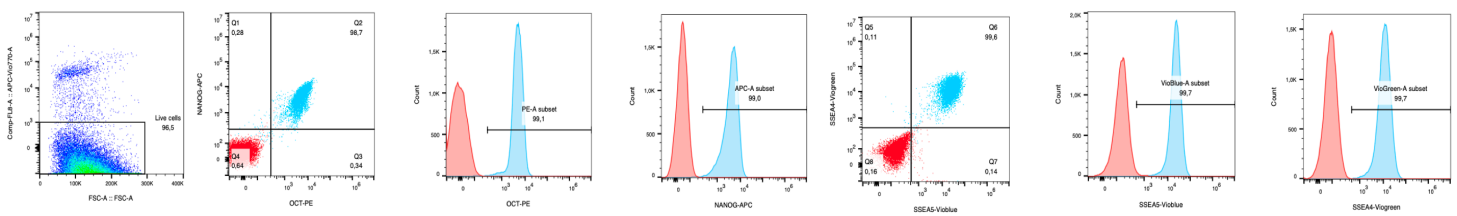
D Recombinant FGF-2 STAB® 100 ng/mL Every Day Feeding



E Recombinant FGF-2 STAB® 50 ng/mL Every Other Day (EOD) Feeding



F Recombinant FGF-2 STAB® 100 ng/mL Every Other Day (EOD) Feeding



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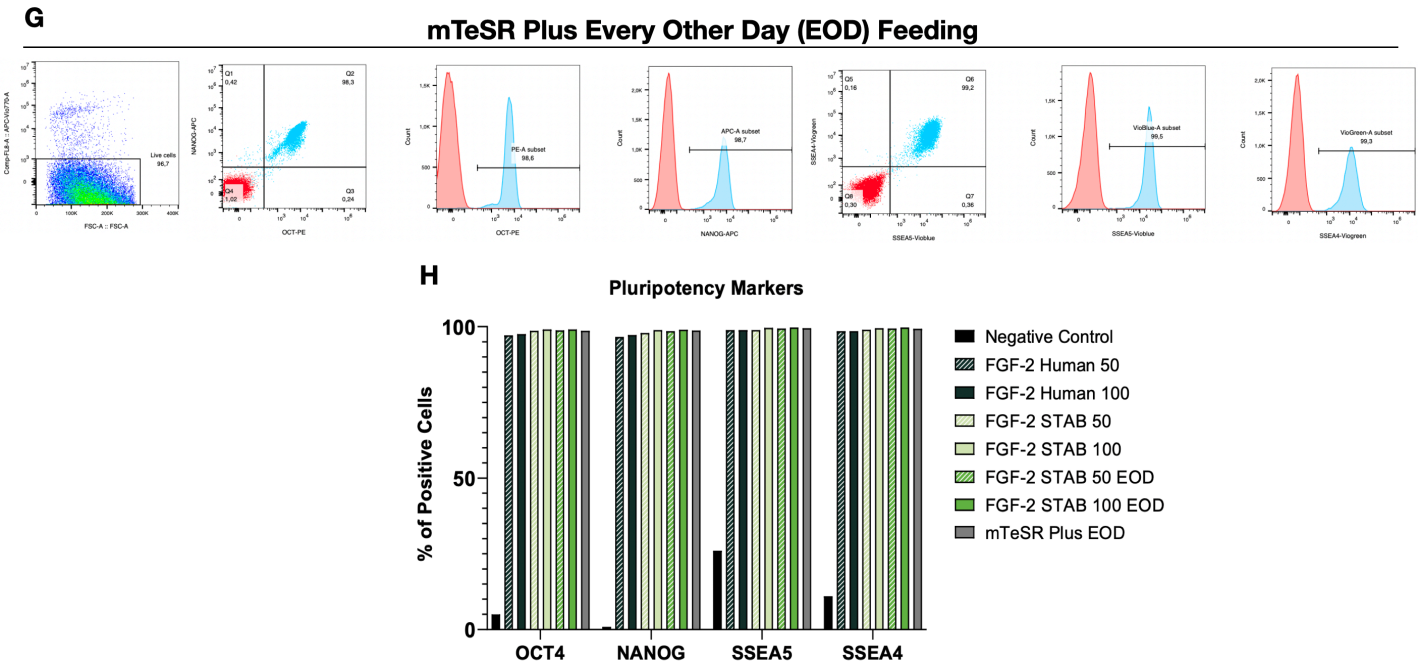

Fig.3 Detection of pluripotency markers by flow cytometry. (A-G) Flow cytomertic analysis of permeabilised iPSCs, exposed to antibodies against OCT 4, NANOG, SSEA5, and SSEA4. All cytometry profiles for the different markers are indicated for each condition (Human Recombinant FGF-2 at 50 and 100 ng/mL with media feeding every day; Recombinant FGF-2 STAB® at 50 and 100 ng/mL with both media feeding every day, and every other day -EOD-, and mTeSR Plus with EOD media feeding. (H) Quantification of positive cells for each pluripotent marker with different media and growth factor conditions, in comparison with iPSC cultured in media with no supplements.

FGF-2 STAB® Maintains Pluripotency Marker Expression for iPSCs Culture, under different Growth Factor Concentrations and Feeding Conditions

Following the cell morphology and proliferation analysis, the effects and capacity of FGF-2 STAB® to maintain high quality iPSC cultures under different concentrations and media feeding regimes, was assessed by determining the expression of key markers of pluripotency. Flow cytometry was employed to rigorously define the level of pluripotent marker expression among the multiple iPSC culture conditions, in a quantitative manner. The panel consisted of two nuclear markers (OCT4 and NANOG), and the cell surface markers (SSEA4 and SSEA5). This panel was chosen as those markers are master regulatory transcription factors necessary for the maintenance of iPSCs, and are key indicators of undifferentiated cells. For the flow cytometry experiments, iPSC cultures were dissociated to single cells, fixed and permeabilized, so that the nuclear markers OCT4 and NANOG are accessible to the antibodies while preserving the surface markers SSEA4 and SSEA5. Data analysis show how iPSCs are strongly positive for the 4 markers (with expression levels always above 95%) among all the conditions (Fig 3. A-G) corresponding to: cell media supplementation with Human Recombinant FGF-2 (at 50 and 100 ng/mL) and every day feeding (Fig.3 A-B), with FGF-2 STAB® (at 50 and 100 ng/mL) and both every day (Fig.3 C-D) and every other day (EOD) feeding (Fig.3 E-F), and with mTeSR Plus with EOD feeding (Fig.3 G). More specifically, when “wild-type” human recombinant FGF-2 was used to supplement

StemFit media and feeding cells every day, iPSCs were positive for all the markers with both the lower (50ng/mL) concentration: OCT4 (> 97%) NANOG (> 96%) SSEA5 (> 98%) and SSEA4 (> 98%) and the higher (100ng/mL) concentration: OCT4 (> 97%) NANOG (> 97%) SSEA5 (> 98%) and SSEA4 (> 98%). Notably, results followed the same trend when the thermostable FGF-2 STAB® was used to supplement the media. For this case, the pluripotency markers showed even higher levels of expression with both the lower (50 ng/mL) concentration: OCT4 (> 98%) NANOG (> 97%) SSEA5 (> 98%) and SSEA4 (> 99%) and the higher (100 ng/mL) concentration: OCT4 (> 99%) NANOG (> 98%) SSEA5 (> 99%) and SSEA4 (> 99%). To functionally investigate how the stability of FGF-2 STAB® allows to maintain healthy pluripotent phenotypes on iPSC cultures even when media is changed every other day (EOD), the same marker expression analysis were performed on cells following this reduced feeding regime. Interestingly, cells were positive to all the markers in almost identical levels compared to the daily media change. In particular, FGF-2 STAB® successfully sustained iPSC in their pluripotent state with high marker expression levels after following EOD feeding schedules at both 50 ng/mL concentration: OCT4 (> 98%) NANOG (> 98%) SSEA5 (> 99%) and SSEA4 (> 99%) and with the higher (100 ng/mL) concentration: OCT4 (> 99%) NANOG (> 99%) SSEA5 (> 99%) and SSEA4 (> 99%). These results were further validated by observing the big differences on the marker expression levels of cells when cultured with StemFit Basic media with no supplements (Fig 3. H). Under this condition and due to the lack of growth factors and essential signalling components, the expression levels were very low (always < 10%) for all the pluripotency markers. More importantly, the benefits of FGF-2 STAB® for providing constant signalling levels, and achieving sustained pluripotency of iPSCs when supplemented with this growth factor, were further supported by the quantified marker expression comparison against cells grown with mTeSR Plus under the same EOD media change schedules (Fig. 3 G and H). All together, these results indicate that FGF-2 STAB® presents a superior performance for culturing iPSCs, and allows to explore reduce media feeding -including weekend-free schedules- without compromising the quality and pluripotency of the cells in culture.

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Conclusion & Future Directions

The stability of Fibroblast Growth Factor 2 (FGF-2) in induced pluripotent stem cell (iPSC) cultures emerges as a critical factor with far-reaching implications in various domains of biomedical research and clinical applications. The sustained stability of FGF-2 within iPSC cultures not only ensures the maintenance of pluripotency but also facilitates robust expansion and differentiation capabilities. This stability provides a foundation for reliable and reproducible experimental outcomes, which are essential for advancing our understanding of stem cell biology and harnessing the full potential of iPSCs.

Furthermore, the implications of FGF-2 stability underscore the importance of ongoing efforts to refine culture conditions and optimize growth factor supplementation strategies. By addressing challenges related to FGF-2 degradation and variability, researchers can unlock the full potential of iPSC technology and accelerate its translation into practical applications in tissue engineering, regenerative medicine, and cell therapy.

Moreover, the use of the plant-derived recombinant FGF-2 STAB® employed in this study, facilitates the development of customized media solutions that are more flexible in addressing variabilities inherent in iPSC cultures, while allowing for a sustainable sourcing of materials, and reducing the expenses in cell culture reagents.

By fine-tuning growth factor concentrations and supplementation regimens, researchers can adapt culture conditions to meet specific experimental requirements and overcome challenges associated with donor variability or cell line characteristics.

In essence, the stability of FGF-2 STAB®, coupled with innovations such as the bio-production of recombinant proteins in plant systems, represents a cornerstone in the advancement of iPSC-based methodologies in a cost-effective, sustainable and customizable manner.

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