Improving protein delivery of Fibroblast Growth Factor-2 from bacterial Inclusion Bodies used as cell culture substrates

Joaquin Seras-Franzoso [1, 2, 3 *], Karl Peebo[4], Elena García-Fruitós [3, 2, 1], Esther Vázquez [1, 2, 3], Ursula Rinas [5, 6], Antonio Villaverde [1, 2, 3]

[1] Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain

[2] Department de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain

[3] CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Bellaterra, 08193 Barcelona, Spain

[4] Competence Centre of Food and Fermentation Technologies, Akadeemia tee 15a, 12618 Tallinn, Estonia

[5] Institute of Technical Chemistry-Life Science, Leibniz University of Hannover, D-30167 Hannover, Germany

[6] Helmholtz Centre for Infection Research, Inhoffenstraße 7, D-38124 Braunschweig, Germany

***** Corresponding author

Joaquin.Seras@uab.cat

Abstract:

Bacterial inclusion bodies (IBs) have been recently used to generate biocompatible cell culture interfaces with diverse effects on cultured cells such as cell adhesion enhancement, stimulation of cell growth or induction of mesenchymal stem cell differentiation. Additionally, novel applications of IBs as sustained protein delivery systems with potential applications in regenerative medicine have been successfully explored. In this scenario, with IBs gaining significance in the biomedical field, the fine tuning of this functional biomaterial is crucial. In this work, the effect of temperature on FGF-2 IB production and performance has been evaluated. FGF-2 has been overexpressed in *Escherichia coli* at 25 °C and 37 °C obtaining IBs with differences in size, particle structure and biological activity. Cell culture topographies made with FGF-2 IBs biofabricated at 25 °C showed higher levels of biological activity as well as a looser supramolecular structure, enabling a higher protein release from the particles. In addition, the controlled use of FGF-2 protein particles enabled the generation of functional topographies with multiple biological activities being effective on diverse cell types.

Keywords: Inclusion bodies, protein release, drug delivery, nanomedicine, surface functionalization

Introduction:

Bacterial Inclusion Bodies (IBs) are protein particles, ranging from 50 nm to 1 micron [1], produced by the deposition of polypeptides under cell stress conditions such as the observed in recombinant protein production processes [2]. In this situation, the strong induction of the protein expression system and the subsequent abnormal amount of newly synthesized polypeptides overload the protein quality control network driving non-folded, partially folded and properly folded proteins to aggregate through a stereospecific process [3]. As a result, highly pure structures capable to retain a certain degree of biological activity can be easily isolated in a cost effective manner [4]. Moreover, IBs have exhibited a high mechanical plasticity being their physicochemical features regulatable by the proper choice of the producing strain's genetic background [5-7]. In addition, the particle size and the conformational quality of the IB forming protein can be tuned by modulating operational variables such as culture temperature or harvesting time as well as engineering the protein construct by the addition of distinct peptide linkers in fusion proteins [8-10].

In the last decade, several new applications of bacterial IBs have been developed from their straightforward use as immobilized biocatalysts [11;12] to their application as a model to study amyloid-related diseases [13;14]. Nevertheless, it is in the biomedical field in which bacterial IBs have recently made an important appearance. These protein particles have been shown to provide positive mechanical stimuli when randomly deposited on cell culture surfaces rendering enhanced cell adhesion and proliferation in several cell lines [15;16] as well as triggering cell differentiation of mesenchymal stem cells towards osteoblasts [17]. In addition, when IBs formed by proteins with specific biological activities such as the enzymes catalase and dihydrofolate reductase, the Hsp70 chaperone, and leukemia Inhibitory factor [18], or the structural protein Keratin-14 [19] were added to the cell media of challenged cell cultures, IBs could complement missing activities by releasing sufficient amounts of soluble and functional protein. A part from this passive protein delivery IBs have recently shown able to be engineered

by the incorporation of self cleaving peptides to obtain active protein delivery platforms [20].

On the other hand, FGF-2 is a pleiotropic cytokine involved in diverse processes such as cell proliferation, cell migration, cell differentiation, integrin expression and embryonic development among others [21]. This protein has been described in four distinct isoforms, one Low Molecular Weight (LMW) protein (18 KDa) and three High Molecular Weight (HMW) forms (22, 22.5 and 24 KDa) [22]. These HMW forms share the same sequence as the, LMW but present an extra peptide that has been described as a nuclear localization signal. Thus, the three HMW FGF-2 forms act at the nuclear level while the LMW form is mainly cytoplasmatic [23]. In this regard, the 18 KDa FGF-2 is secreted and triggers signaling cascades via its interaction with the FGF-2 receptor [24], differentiating FGF-2 action in an extracellular triggered activity (LMW) and an intracellular action (HMW). Interestingly, FGF-2 has been previously shown as a protein able to form biological active IBs being able to stimulate cell growth in mouse fibroblast NIH3T3[25] .

In this contribution, we explore the effect of temperature on the IBs biofabrication process, focusing on the structure and biological activity of FGF-2 IBs with a final application in the cell therapy context. The broad range of activities of FGF-2 renders this protein an ideal model to optimize IBs as a delivery system in *in vitro* cell cultures from a cost-effective process.

Materials and Methods

Bacterial strains and plasmids.

The *E. coli* strain BL21 (DE3) and *E. coli* K-12 derivative MC4100 (araD139 [argF-lac]U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR Strr) were used for IBs fabrication of the unfused human fibroblast growth factor 2, hFGF-2 (Low Molecular Weight isoform, 155 amino acid, 18 KDa) and the fusion protein VP1GFP, respectively. FGF-2 was cloned into a pET29c(+)-hFGF-2 vector with no additional pull-down tags [26], while VP1GFP was placed in the pTVP1GFP vector under the control of a P_{TRC} promoter [27]. VP1GFP consisted of a fusion protein formed by GFP linked to the VP1 domain. VP1 corresponds to a capsid protein of the foot-and-mouth disease virus acting as an aggregation domain and facilitating the deposition as IBs of the otherwise highly soluble GFP.

IB production and purification

IBs were produced in shake flasks in Luria Bertani (LB) medium. Bacterial inoculum were adjusted at a starting optical density at 550 nm (OD_{550}) of 0.05 and cultured at 37 °C and 250 rpm. Once the cultures reached an OD_{550} between 0.5 and 0.7, isopropyl-D-thiogalactoside (IPTG) was added at 1 mM in order to induce gene expression and the subsequent protein deposition as IBs. Protein production was obtained at 250 rpm during 3h, at either 25 °C or 37 °C in the case of FGF-2, and at 37 °C in the case of VP1GFP.

After protein production, bacterial IBs were isolated by a combination of enzymatic and soft mechanical cell lysis, followed by extensive sample washing in presence of mild detergents. Firstly, lysozyme was added at 1 µg/mL (final concentration) and incubated at 37 °C under agitation at 250 rpm for 2 h. Next, several cycles of freeze/thaw were performed in order to disrupt the partially digested bacteria to release the IBs. IBs were then washed in 0.4 % Triton X-100 (v/v), 1 h at RT and in 0.025 % NP40 (v/v), 1h 4 °C. Then samples were treated with DNase at 0.6 µg/mL (final concentration), for 1h at 37 °C. DNase treatment and washing with detergent were carried out in order to remove

bacterial DNA and cell membrane contaminants. Finally, additional washing steps with PBS were applied to the IB samples. Furthermore, IBs were checked after cell disruption by plating 100 µL of sample on LB plates, incubating overnight at 37°C. This checkpoint was included to guarantee the absence of viable bacteria in the final IB preparation.

IB proteins were quantified by Western Blot using the Quantity One® software (Bio-Rad, CA, USA). Sample protein amount was inferred from standard curves with known amounts of each protein.

IB size determination

Particle size of FGF-2 IBs produced at 37 °C and 25 °C was analyzed using a dynamic light scattering (DLS) instrument (Malvern, Nanosizer Z, Worcestershire, UK). IBs were resuspended in ultra-pure water and sonicated for 1 min at 40 W in 0.5'' pulses to facilitate the formation of an appropriate particle suspension. DLS measurements were carried out in triplicate averaging 3 single measurements.

IB re-solubilization assay

100 µg of FGF-2 IBs, produced at both 25 °C and 37 °C, were resuspended in 1 mL of cell medium, D-MEM with 1% FBS (v/v) and incubated at 37 °C for 24h under gentle agitation. After incubation, samples were centrifuged for 15 min at 15000g. Soluble and insoluble fractions were quantified by Western Blot analysis and the relative amount of released protein determined.

Cell culture

NIH3T3 cells were routinely maintained in D-MEM supplemented with FBS 10% (v/v) and 2 mM L-glutamine at 37 °C and 10% CO_2 in a humidified incubator. PC12 were routinely cultured on Poly-Lys treated plates in D-MEM supplemented with FBS 10% (v/v) at 37 °C and 10% CO_2 in a humidified incubator. HeLa and HepG2 cells were maintained in MEM alpha supplemented with FBS 10% (v/v) and 2 mM L-glutamine at 37 °C and 5% CO_2 in a humidified incubator, and 1671 cells were routinely cultured in

D-MEM supplemented with FBS 10% (v/v), 2 mM L-glutamine and insulin 0.005 mg/mL at 37 °C and 5% CO_2 in a humidified incubator.

Cell proliferation assay

Biological activity of FGF-2 IBs was assayed by measuring cell proliferation of the murine cell line NIH3T3. Non-treated 96-well plates (Costar®; Corning, MA, USA) were decorated with IBs. Briefly, IBs were resuspended in cell culture medium with 1% FBS (v/v) at different concentrations. Then, 50 µL of IB suspension were added per well, resulting in protein amounts ranging from 0.2 ng to 0.2 µg per well. These plates were incubated at 4 °C ON in order to allow IB sedimentation. IBs formed by the fluorescent protein VP1GFP were included as negative control (irrelevant IBs = IR IBs) for the FGF-2 biological activity and commercial soluble FGF-2 at 0.01 µg / mL was used as a positive control of the test. Due to the higher specific activity of the commercial protein lower concentrations were needed in order to stimulate NIH3T3 cells growth. Noteworthy that 24h prior to IBs addition, NIH3T3 cultures were exposed to a serum starvation stress by reducing FBS from 10% to 1%. 4 X 10^3 NIH3T3 cells were seeded per well and cultured for 48 h at 37 °C and 10% CO_2 in a humidified incubator.

Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, using the EZ4U kit (Biomedica, GmbH, Vienna, Austria) following manufacturer's instructions. Measures were acquired in a Victor 3V 1420 (Perkin Elmer™; MA, USA) reader. The assay was carried out in triplicate and variability analyzed by t-test.

HeLa, PC12, HepG2 and 1671 cell growth was analyzed following the same procedure described above using 0.2 µg IBs/well to fabricate the IB topography. The starting cell number was adjusted to 2.5 X 10^3, 4 X 10^4, 7 X 10^3 and 2 X 10^4 cells, respectively, and measures were taken at 48 and 72 h. Results were shown as the relative cell number with respect to the cells growing in un-supplemented medium.

PC12 differentiation assay

The effect of FGF-2 IBs on PC12 differentiation was analyzed by measuring the neurite outgrowth of PC12 cell cultures in DMEM with 1% FBS (v/v) and 2mM L-glutamine, after 7 days of the IB addition. 10^3 cells/cm^2 were seeded onto 24 well plates (BD Falcon™; Becton Dickinson, NJ, USA) previously coated with collagen. Bright field images of cells were acquired and analyzed using ImageJ software. Briefly, images were transformed to binary masks by adjusting threshold intensity at 165 points. These masks were treated by using fill holes, erode and dilate commands in order to obtain individual cell shapes completely filled. After the preprocessing, skeletonize command was applied resulting in 1 pixel width cell skeletons. The neurite growth values shown correspond to the length measure of at least 85 individual cells in presence of FGF-2 25°C IBs, FGF-2 37°C IBs, and the negative control with no IBs. In the case of the positive differentiation control (in presence of soluble Nerve Growth Factor, NGF) 25 individual cells were analyzed. Relative cell numbers were obtained by representing the number of cells obtained for each condition at every range of the neurite outgrowth distribution as a percentage of the total amount of cells analyzed per each condition.

Results:

As there are multiple biological functions of FGF-2, we focused our study in two main activities with potential interest in the biomedical and cell culture fields, namely the promotion of cell proliferation and the induction of cell differentiation.

FGF-2 produced for 3 hours at either 25 °C or 37 °C both formed IBs. Once isolated, these protein nanoparticles were deposited forming random nanotopographies that were able to stimulate NIH3T3 proliferation in serum-starved cell cultures, in a dose-dependent manner (Figure 1). Interestingly, this effect was observed with a higher extent for FGF-2 IBs produced at 25 °C. Moreover, cells cultured on control IBs formed by a VP1GFP protein, irrelevant with respect to cell division (IR IBs), exhibited similar relative cell numbers at all conditions (Figure 1), indicating that the effect of FGF-2 IBs was provided by the specific biological activity of these IBs rather than a mechanical stimuli carried out by the IB topography, as it has been described in other cases [16]. This fact suggests the presence of properly folded protein embedded in the IB scaffold, being higher for IBs produced at lower temperatures.

Nevertheless, the potential use in the biomedical field of growth factors in IB form is considerably enriched by the various biological roles played by these factors. In the present work, the ability of FGF-2 to induce an early neuron-like differentiation of PC12 cells was also analyzed to demonstrate the ability of these IBs to act as depot of the multiple activities of the forming protein. As one of these activities, neurite outgrowth of PC12 cells growing in presence of FGF-2 IBs produced at 25 °C and 37 °C, as well as NGF (positive control) and without IBs (negative control) was investigated (Figure 2a-d). The neurite length measurements revealed a clear tendency of PC12 cells to generate longer neurites in the presence of FGF-2 IBs compared to the negative control. Being FGF-2 IBs produced at 25 °C the ones stimulating a higher extent of neurite outgrowth (Figure 2e).

In order to investigate the differences in the activity observed for the FGF-2 IBs produced at different temperatures, the particle size was determined (Figure 3a).

Protein particles produced at 25 °C were nearly 100 nm larger in diameter than the ones produced at 37 °C. In addition, after incubating equal amounts of 25 °C and 37 °C IBs in cell culture media, the recovery of soluble FGF-2 was higher in the IBs produced at the lowest temperature (Figure 3b). These results suggest that the higher activity detected for the IBs produced at 25 °C could be due to a higher protein release from the IB surface.

Finally the activity of FGF-2 scaffolds was tested with other cell lines. A potential effect of FGF-2 IBs on different cell lines grown at different conditions would increase the applicability of FGF-2 IBs in the biomedical field. To this end, cell proliferation was measured after culturing cells for 48 and 72h on FGF-2 IB scaffolds. This test was carried out with the more active particles produced at 25 °C on four cell types with different biological requirements, namely PC12 (rat pheochromocytoma adrenal medulla cells), 1675 (uterus endometrium carcinoma cells), HeLa (human epithelial carcinoma cells) and HepG2 (human hepatocellular liver carcinoma cells). Interestingly, all the analyzed cell lines exhibited higher relative cell numbers when cultured on FGF-2 IBs comparing to cells growing on IR IBs (Figure 4). Noteworthy, in order to get a finer response to the FGF-2 activity the experiment was carried out under serum deprivation conditions (1% FBS (v/v). Under this serum reduced environment the addition of FGF-2 in the IB form resulted in significant differences on cell growth on the four cell lines.

Discussion:

Previous studies showed that FGF-2 IBs were active and useful in bioengineering and nanomedicine in form of both nanopills and bioactive scaffolds[25] . In the present work, the temperature-mediated tuning of these particles was explored showing a significant importance of the production design in the resulting particles' performance.

In this regard, it is important to stress that IB structure and composition should be evaluated case per case depending on the final application, and biofabrication conditions adjusted accordingly to obtain the major extent of biological activity and therefore to increase the potential of the modified cell culture surfaces. We have here observed that the IBs produced at lower temperature possess higher potential to make available bioactive protein by showing a higher content of properly folded protein. This property would be reinforced by a looser IB supramolecular structure enabling the re-solubilization of a higher amount of protein. These two features are probably linked, since a higher percentage of protein in its native-like form would favor the solubility of the extracted polypeptides, avoiding partially unfolded polypeptides to go through a more complex refolding process and therefore preventing re-aggregation. These results are in agreement with recent studies in which native-like protein content in IBs was analyzed and related to the IB production temperature [8;28]. These studies also evidenced a higher protein release in their biologically active form when IBs were produced at lower temperatures, leading to simpler re-solubilization procedures for recombinant proteins with tendency to from insoluble aggregates [8;28]. These properties also apply for the straightforward application of bioactive IBs in the cell culture context.

On the other hand, the cellular location of protein action has also to be considered. Arese and co-workers demonstrated that cell proliferation of the NIH3T3 cell line at low serum concentrations is stimulated by the HMW forms of the FGF-2 protein, being associated with a nuclear action [23]. In addition they showed that a chimeric LMW FGF-2 (fused to an NLS peptide) was capable to emulate HMW forms activity [23]. In

our study, FGF-2 IBs when provided as modified cell culture surfaces were able to enhance cell growth under serum starvation conditions, considering that this activity has been previously linked to a nuclear activity and our IBs are formed by the cytosolic LMW form of the protein it is feasible to speculate that FGF-2 IBs were internalized, as it have been described for other IB types [18;19], delivering the forming protein to the nucleus, the way in which this process takes place remains still unclear and deserves further studies. However, particle size would be an important parameter to evaluate being, in the case of IBs, closely related with the protein production temperature. Particle size has been inversely associated with cell internalization efficiency [29]. Interestingly, the bigger IB size produced at 25°C seems to be compensated by the higher availability of bioactive proteins. Nevertheless, this fact cannot be extended to IBs formed by other proteins, since each protein type would present a different supramolecular structure given by the pattern of interactions through hydrophobic exposed patches available in the interacting polypeptides.

Finally, the use of biologically active IBs formed by essential growth factors, cytokines or other essential proteins presents several advantages in comparison to other immobilization methods on cell culture surfaces, in which soluble protein would be required [30-32]. IB production and isolation is significantly cheaper since the naturally self-assembly of the over-expressed protein into insoluble particles facilitates enormously the downstream process [33]. In addition, the proper choice of the *E. coli* genetic background and protein production process variables allow a fine tuning of the IB physicochemical properties and the conformational quality of the embedded polypeptides [5;34-36].

Conclusions:

The present study strongly supports the use of bacterial IBs as a compatible biomaterial for protein immobilization on cell culture surfaces and subsequent release of biologically active protein. We have focused on the effect of temperature on the final properties of the generated interfaces. In this regard, modified surfaces formed by IBs of growth factors such as FGF-2 exhibited a biological activity able to be fully modulated by their production temperature with an impact also on the particle size. Such plastic bioactive material could be extremely useful for the accurate functionalization of devices with applications in several biological interfaces. More specifically, FGF-2 IB topographies are a good model to study growth factors and cytokines in IB-based approaches for drug delivery, but they are also a potential tool *per se*. Results obtained in the present study confirm that FGF-2 IB-modified surfaces can act as a multifunctional platform for a broad range of target cell types.

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Figure 1

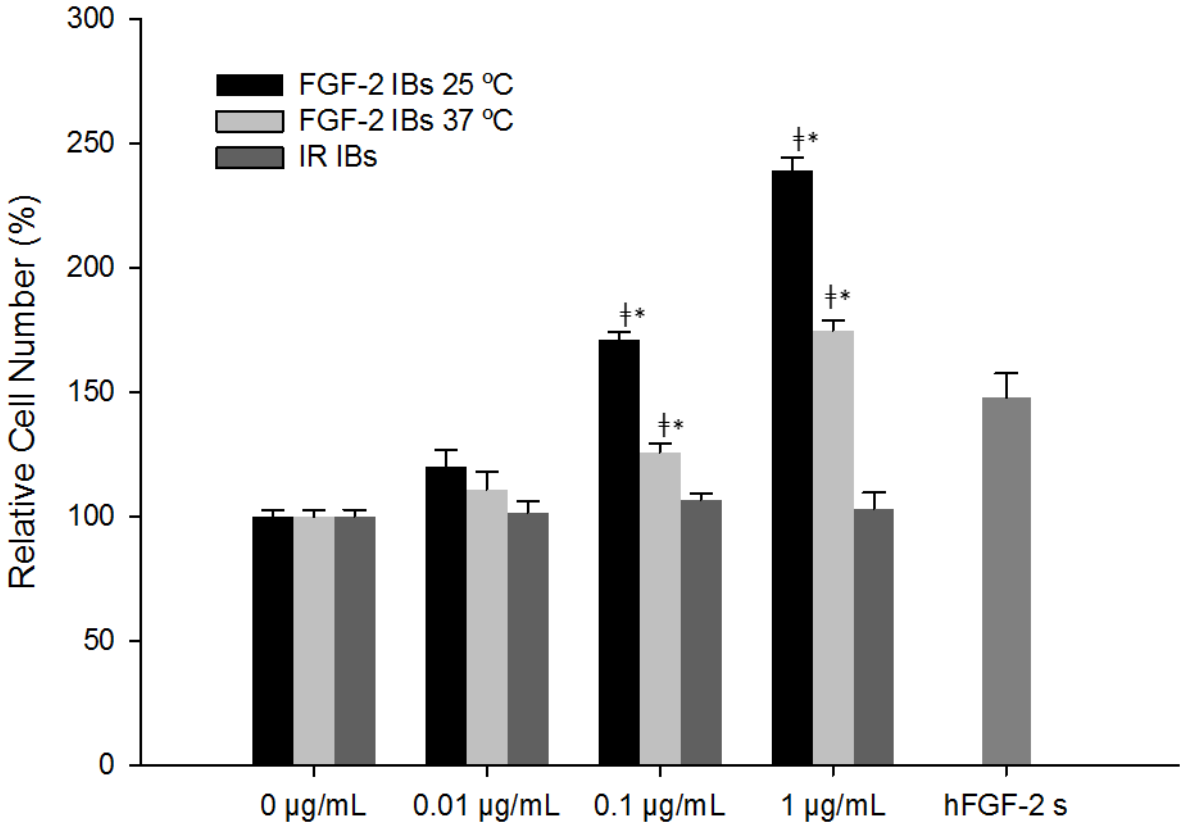
300
250
200
150
100
50
0
Relative Cell Number (%)
FGF-2 IBs 25 ºC
FGF-2 IBs 37 ºC
IR IBs
‡*
‡*
‡*
‡*
0 µg/mL
0.01 µg/mL
0.1 µg/mL
1 µg/mL
hFGF-2 s

Figure 2

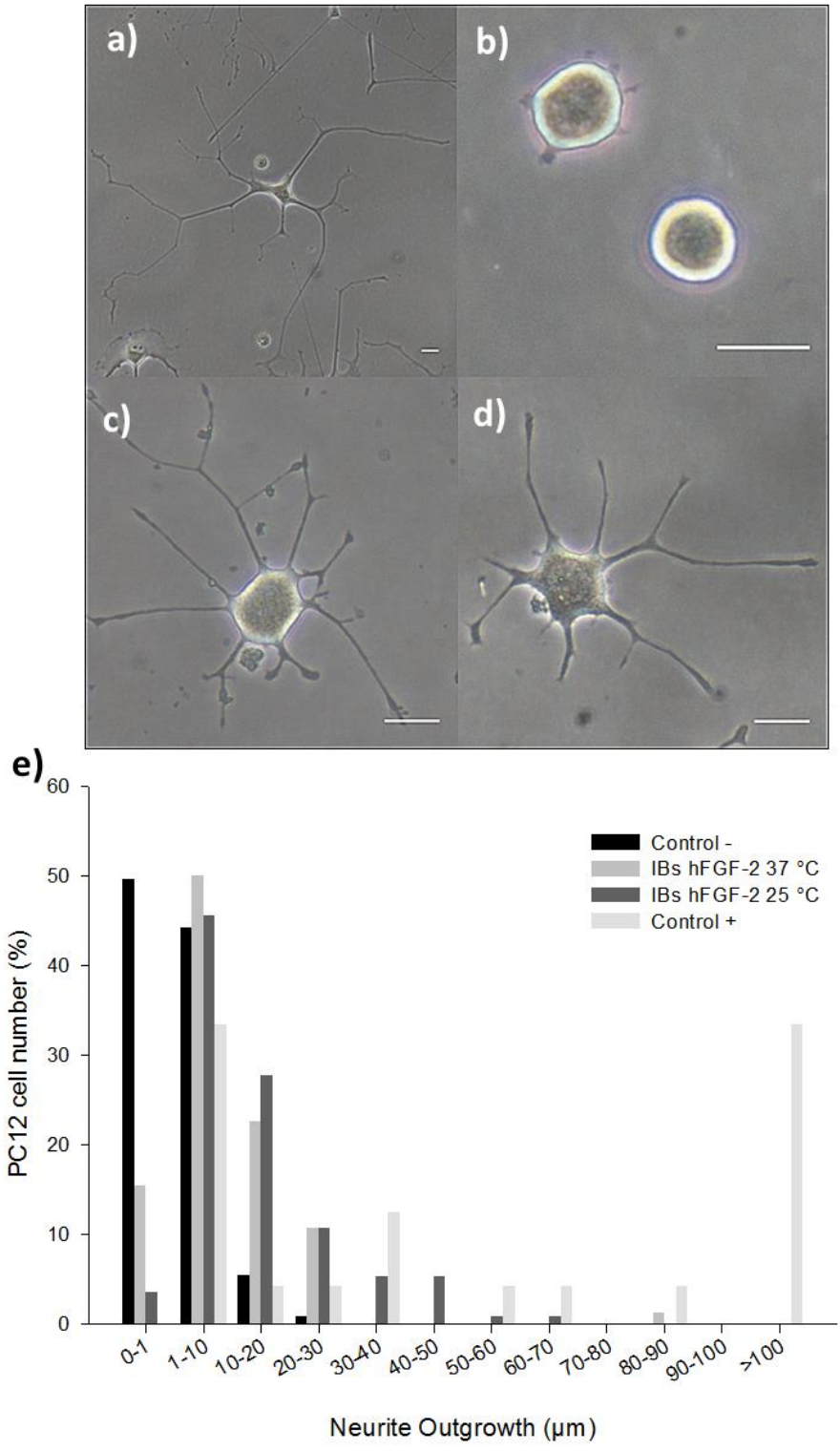
a)
b)
c)
d)
e)
PC12 cell number (%)
Neurite Outgrowth (µm)
Control -
IBs hFGF-2 37 °C
IBs hFGF-2 25 °C
Control +
0
10
20
30
40
50
60
0-1
1-10
10-20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
90-100
>100

Figure 3

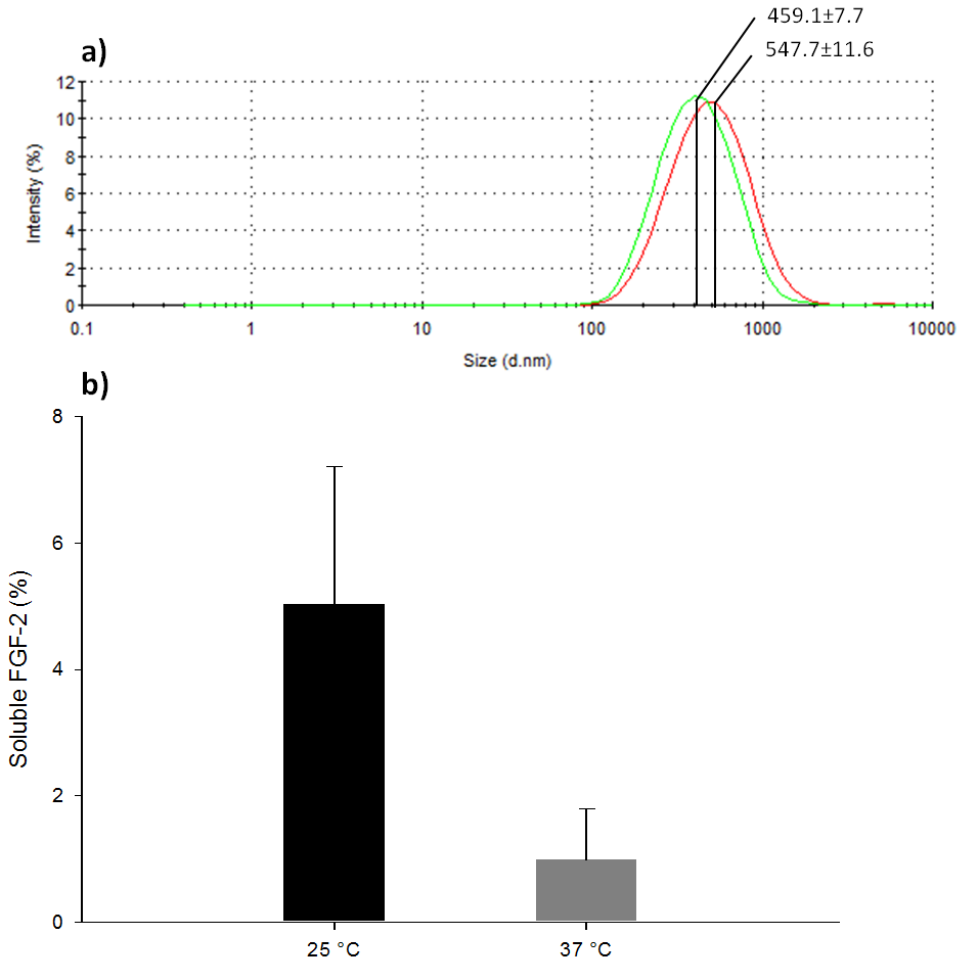

Figure 4

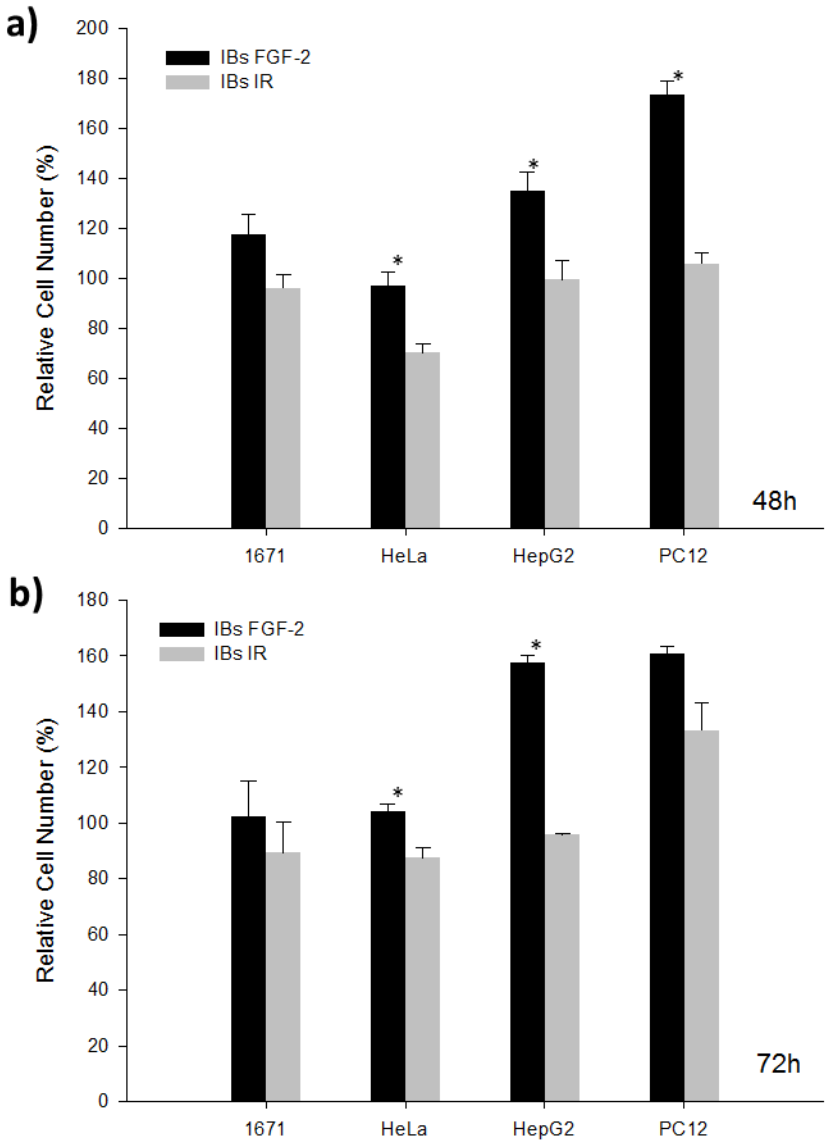
a)
IBs FGF-2
IBs IR
Relative Cell Number (%)
200
180
160
140
120
100
80
60
40
20
0
*
*
*
48h
1671
HeLa
HepG2
PC12
b)
IBs FGF-2
IBs IR
Relative Cell Number (%)
180
160
140
120
100
80
60
40
20
0
*
*
72h
1671
HeLa
HepG2
PC12

Figure Legends

Figure 1. Biological effects of FGF-2 IBs on NIH3T3 cell cultures. Growth of NIH3T3 cells cultured on FGF-2 IBs produced at 25 °C and 37 °C and IR IBs (negative control) after 48h of incubation. Soluble FGF-2 (positive control) is represented in the last column (0.01 µg/mL). Significant differences ($p \leq 0.05$) between pairs are indicated: FGF-2 IBs 25°C and 37°C (?) and FGF-2 IB and VP1GFP (negative control) **(*).**Standard error is represented by black lines above the bars.

Figure 2. Biological effects of FGF-2 IBs on PC12 cell cultures**.** Panel a,b,c and d: PC12 cells growing in presence of : a) soluble NGF (positive control), b) regular medium (negative control), c) FGF-2 IBs (25 °C) and d) FGF-2 IBs (37 °C) respectively, 5 µm white scale bars are showed in all the micrographs. Panel e: neurite outgrowth distribution of PC12 cells cultured on the aforementioned conditions.

Figure 3. Physicochemical characterization of FGF-2 IBs. Panel a: size distribution of FGF-2 IBs 25 °C (red line) and 37 °C (green line). Units to represent size – diameter in nm. Panel b: percentage of soluble FGF-2 recovered after 24h incubation in cell culture media of FGF-2 IBs produced at 25 °C (black bar) and 37 °C (grey bar). Standard error is represented by black lines above the bars.

Figure 4. Differential effects of FGF-2 IBs on cell growth. Cell growth of 1671, HeLa, HepG2 and PC12 cells cultured on FGF-2 IBs (25 °C) and IR IBs scaffolds at 48 h (panel a) and 72 h (panel b). Significant differences ($p \leq 0.05$) between the FGF-2 IB and VP1GFP (negative control) are indicated (*). Standard error is represented by black lines above the bars.

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