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Improved scaffold biocompatibility through anti-Fibronectin aptamer functionalization

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Title: Improved scaffold biocompatibility through anti-Fibronectin aptamer functionalization

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Abstract: Protein adsorption is the first and decisive step to define cell-biomaterial interaction. Guiding the adsorption of desired protein species may represent a viable approach to promote cell activities conducive to tissue regeneration. The aim of the present study was to investigate whether immobilized anti-Fibronectin aptamers could promote the attachment and growth of osteoblastic cells.

Polyethyleneglycole diacrylate/thiolated Hyaluronic Acid hydrogels (PEGDA/tHA) were coated with anti-Fibronectin aptamers. Hydrogel loading and Fibronectin bonding were investigated, through spectrophotometry and Bradford assay. Subsequently, human osteoblasts (hOB) were cultured on hydrogels for 10 days in 2D and 3D cultures. Cells were monitored through microscopy and stained for focal adhesions, microfilaments and nuclei using fluorescence microscopy. Samples were also included in paraffin and stained with Hematoxylin-Eosin. Cell number on hydrogels was quantitated over time. Cell migration into the hydrogels was also studied through Calcein AM staining. Aptamers increased the number of adherent hOBs and their cytoplasm appeared more spread and richer in adhesion complexes than on control hydrogels. Viability assays confirmed that significantly more cells were present on hydrogels in the presence of aptamers, already after 48 hours of culture. When hOBs were encapsulated into hydrogels, cells were more numerous on aptamer-containing PEGDA-tHA. Cells migrated deeper in the gel in the presence of DNA aptamers, appearing on different focus planes. Our data demonstrate that anti-Fibronectin aptamers promote scaffold enrichment for this protein, thus improving cell adhesion and scaffold colonization.

## Statement of Significance

We believe aptamers coating of biomaterials is a useful and viable approach to improve the performance of scaffold materials for both research and possibly clinical purposes, because different medical devices could be envisaged able to capture bioactive mediators from the patients' blood and concentrate them where they are needed, on the biomaterial itself. At the same time, this technology could be used to confer 3D cell culture scaffold with the ability to store proteins, such as fibronectin, taking it from the medium and capture what is produced by cells. This is an improvement of traditional biomaterials that can be enriched with exogenous molecules but are not able to selectively capture a desired molecule.

Dear Dr. Murphy,

we would like to thank you further considering our manuscript and for giving us a chance to further improve our work.

Please find the answers to the criticisms of the Editorial Office and the Reviewers listed below.

Editorial Office:

Q: As per our Guide to Authors, please remove "et al." from the references in the reference list and include the names of each of the authors.

A: The reference list was corrected as recommended.

Q: In regards to Figure 2B, statements regarding quantification should also be accompanied by statistical analysis and error bars.

A: The ImageJ quantification was repeated and error bars and statistical analysis were added. Furthermore, an additional graph was inserted to show the correlation between the amount of aptamer used for the functionalization and fluorescence.

Q: It is difficult to see the scale bars in Figure 4D and 4E.

A: Scale bars were added to the images.

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Reviewer #2:

Major comments:

Q: The abstract lacks necessary motivation prior to introducing the aim of the present study.

A: The abstract was modified as recommended.

Q: Figures are commonly mislabeled throughout the text.

A: The manuscript was edited and corrected.

Q: Although dramatically improved from the original submission, numerous issues remain with the quality of writing, proper use of English, and spelling.

A: The manuscript was revised and the quality of language was improved.

Q: Fig. 2B lacks error bars.

A: The ImageJ quantification was repeated and error bars and statistical analysis were added. Furthermore, an additional graph was inserted to show the correlation between the amount of aptamer used for the functionalization and fluorescence.

Q: Fig. 2D: The authors are encouraged to label the gel in a manner that readers can discern differences and avoid the necessity of an overly wordy figure legend.

A: We agree that Fig. 2D was not easy to read and was edited to make it clearer.

Q: Fig. 3C-D: The relative differences of aptamer binding are more evident at 35  $\mu\text{g}/\text{mL}$  fibronectin compared to 70  $\mu\text{g}/\text{mL}$ . Can the authors comment why higher concentrations of fibronectin might yield similar or lower adsorbed concentrations to aptamer-presenting hydrogels?

A: The reviewer raises an excellent point. A likely explanation is that approximately 3  $\mu\text{g}$  of Fibronectin were able to saturate the aptamer binding capacity in the gels we tested. This is the amount of Fibronectin that was measured on the gels regardless of the protein concentration in the supernatant. A Fibronectin concentration of 70  $\mu\text{g}/\text{mL}$  did not increase the amount of adsorbed protein after 48 hours because all docking points were all possibly already full.

Q: Fig. 4F: the percentage of coverage is so small (0.3%). Were additional studies performed with more cells to perhaps demonstrate the efficacy of adhesion for higher cell concentrations, similar to the in vivo condition? Can the authors comment on why this is so small?

A: We agree with the reviewer that the amount of coverage is very small. However, Fig. 4F reports coverage by ingrowing cells from 2D cultures, which is indeed a limited phenomenon in the

absence of the cytokine/chemokine mediators that are present in a typical in vivo model of implantation. We can hypothesize that the presence of a clot, the inflammatory infiltrate, and possibly a different rate of gel degradation/stability in vivo may have affected cell migration into the scaffold. We did try higher seeding concentrations, but cell ingrowth from 2D cultures on these gels remained quite limited.

Q: What are the units on Fig 6E?

A: Cluster size ( $\mu\text{m}$ ) was normalized by the cluster size in the control group, and the graph therefore has no unit.

Minor comments:

Q: This reviewer found the resubmission particularly difficult to review given the numerous sections of text deleted or moved yet still retained in the document.

A: We apologize for the inconvenience. The conversion of the original word document into a pdf showed also the deleted text. We fixed that to improve the readability of the text.

Q: It appears from the data that binding efficiency of the aptamer, regardless of dosage, was near 100%. If this is true, the authors should clearly state that is the case to bolster the importance of the data.

A: We believe these data are real, and reflect the availability of functional groups in the gel that can form covalent bonds with aptamers. We followed the reviewers suggestions and highlighted this in the manuscript.

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### Reviewer #3

Q: Although the introduction and discussion have been improved in regards of the advantages of this method compared to other strategies, the state of the art still lacks a more exhaustive analysis of the use of aptamers in surface coating. In particular, the authors should explain whether there are previous reports using anti-FN aptamers or not.

A: According to the suggestions of the reviewer, we edited the text and improved the introduction and the discussion sections. We cited all the reports of aptamer-functionalized surfaces, which are few, to the best of our knowledge, and do not include anti-fibronectin aptamers to improve cell adhesion.

Q: If the sequence of the aptamer cannot be disclosed because it belongs to a company, this has to be clearly stated in the manuscript.

A: The aptamer does belong to a company and its sequence cannot be disclosed. We stated this in the manuscript as recommended.

Q: After reading the manuscript as a whole, Fig. 1a seems a bit misleading and does not accurately reflect the data presented. In the absence of aptamers, cells do not bind to the surface due to the low adherence of proteins on the surface. Thus, the figure would be clearer if in the top image (surface without aptamer) serum proteins are shown "floating" and not attached to the surface.

A: We agree with the reviewer that Fig.1A seems to be misleading and following the suggestions, we modified the figure and represented the serum proteins as floating in the environment and not adsorbed on the scaffold.

Q: In the Methods part, section 2.5: hydrogel "selectivity" for fibronectin is not correct. To use the term selectivity in this case the aptamers should be incubated with FN and other proteins, which is not the case. Therefore I suggest the authors to use another terminology, such as "binding activity".

A: We modified the terminology in the manuscript as recommend.

Q: There seems to be some little confusion with the inclusion of new in vitro data. For instance, in the Results section 3.5, the reference to Figure 6C (which actually refers to migration) does not seem correct here. In other sections, Figures are not referenced in appropriate order. Please revise the Results section.

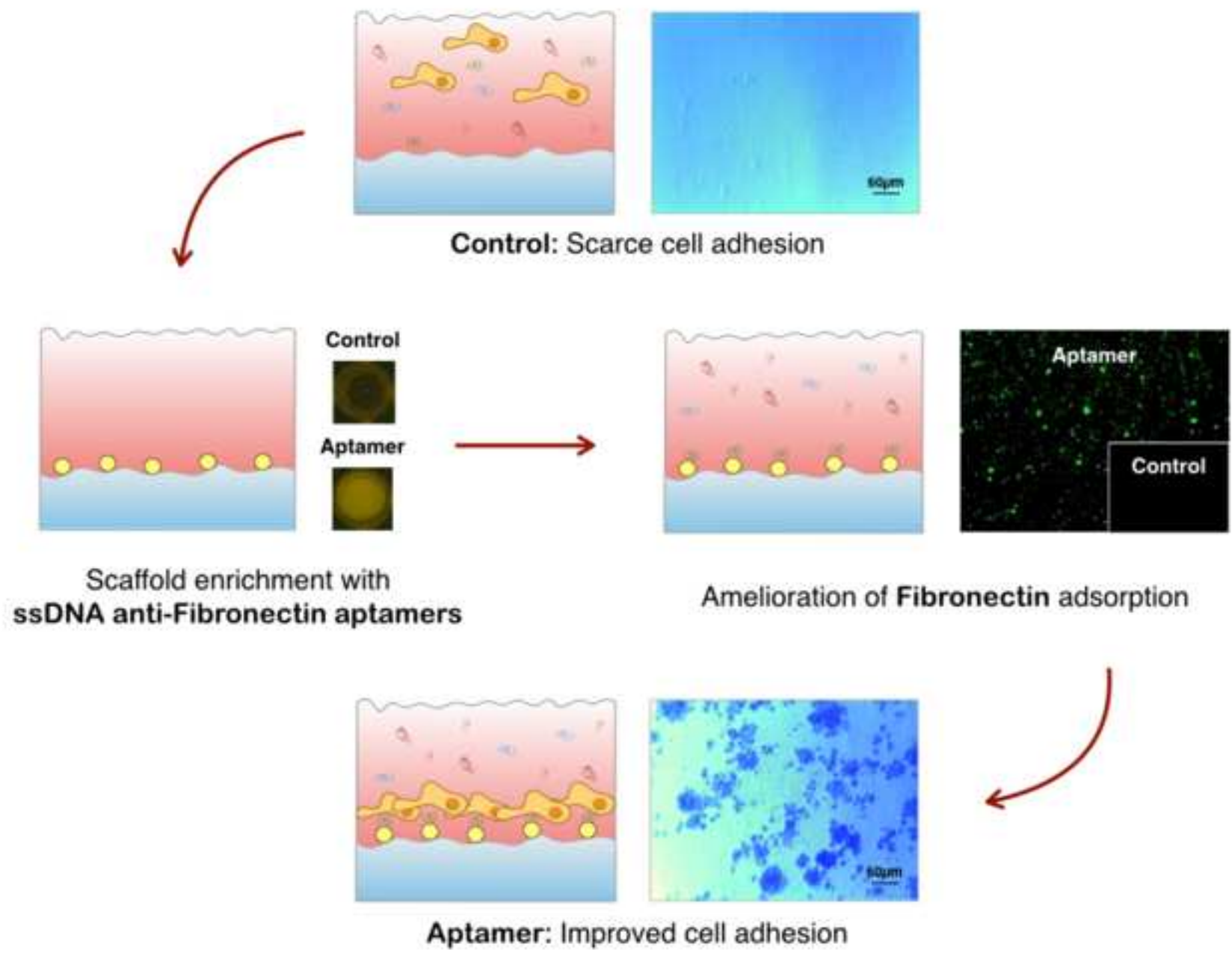
A: We found errors and mislabelled figures in the text. As a consequence, the manuscript was edited and corrected as suggested.

Q: The experiment in the absence/presence of serum (Fig. S2) is an important proof of the necessity of proteins to support cell adhesion and thus of the biofunctionality of the aptamers. This is indicated in the discussion (page 27) but not in the Results section. This relevant experiment should also be indicated in the Results section.

A: The results section was edited as suggested.

Q: The western blot assay is another nice experiment to check the specificity of the aptamers for FN. The bands that appear in Fig S1 could be labeled to identify which band corresponds to which fragment. There is a third, unidentified band. Whereas it is OK not to further examine this band in this work, the sentence "it may be represented by the 120 kDa internal fragment of Fibronectin, possibly for spontaneous proteolysis by serum enzymes..." sounds rather vague if not accompanied by a suitable reference. What does "the internal fragment" mean?

A: The sentence we used was indeed rather obscure. Some previous works (Al-Hazmi et al., 2007) have shown that digestion of Fibronectin with MMP-9 yields a 120kDa fragment which contains the cell-binding domain of Fibronectin. We found no evidence supporting the spontaneous occurrence of this phenomenon in vitro and this remains just a hypothesis that will have to be confirmed in future studies. Clearly, this 120kDa fragment could correspond to another, completely different, protein. We modified the text to make it clearer.



Dear dr. Murphy,

Please find attached the revised manuscript “Improved scaffold biocompatibility through anti-Fibronectin aptamer functionalization” by C. Galli, L. Parisi, M. Piergianni, A. Smerieri, G. Passeri, S. Guizzardi, F. Costa, S. Lumetti, E. Manfredi and GM. Macaluso. The study focuses on the functionalization of hyaluronic acid-based hydrogels with aptamers against fibronectin to improve their biocompatibility. We improved cell responses to hydrogels by enriching them with ssDNA aptamers capable to capture fibronectin from the surrounding environment, the culture medium or blood. We believe this is a useful and viable approach to improve the performance of scaffold materials for both research and possibly clinical purposes, because different medical devices could be envisaged able to capture bioactive mediators from the patients’ blood and concentrate them where they are needed, on the biomaterial itself.

We think this study has been substantially improved thanks to the contribution and the criticisms of the reviewers and is now of potential interest to the readers of Acta Biomaterialia. This manuscript has not been published previously, that it is not under consideration for publication elsewhere, and that if accepted it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the publisher.

Best regards

the authors



# Improved scaffold biocompatibility through anti-Fibronectin aptamer functionalization

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## Abstract

The Protein adsorption is the first and decisive step to define cell-biomaterial interaction. Guiding the adsorption of desired protein species may represent a viable approach to promote cell activities conducive to tissue regeneration. The aim of the present study was

to investigate whether immobilized anti-Fibronectin aptamers could promote the attachment and growth of osteoblastic cells.

Polyethyleneglycole diacrylate/thiolated Hyaluronic Acid hydrogels (PEGDA/tHA) were coated with anti-Fibronectin aptamers. Hydrogel loading and Fibronectin bonding were investigated, through spectrophotometry and Bradford assay. Subsequently, human osteoblasts (hOB) were cultured on hydrogels for 10 days in 2D and 3D cultures. Cells were monitored through microscopy and stained for focal adhesions, microfilaments and nuclei using fluorescence microscopy. Samples were also included in paraffin and stained with Hematoxylin-Eosin. Cell number on hydrogels was quantitated over time. Cell migration into the hydrogels was also studied through Calcein AM staining. Aptamers increased the number of adherent hOBs and their cytoplasm appeared more spread and richer in adhesion complexes than on control hydrogels. Viability assays confirmed that significantly more cells were present on hydrogels in the presence of aptamers, already after 48 hours of culture. When hOBs were encapsulated into hydrogels, cells were more numerous on aptamer-containing PEGDA-tHA. Cells migrated deeper in the gel in the presence of DNA aptamers, appearing on different focus planes. Our data demonstrate that anti-Fibronectin aptamers promote scaffold enrichment for this protein, thus improving cell adhesion and scaffold colonization.

**Keywords:** hydrogels, biomimetics, cell culture techniques, osteoblasts, cell adhesion

## 1. Introduction

Biomaterials, once inserted into the surgical wound, get in contact with blood and spontaneously adsorb plasma proteins ~~based on protein availability and conformation~~ [1,2], ~~which are~~ ~~Proteins~~ are attracted and retained on biomaterial surfaces mostly by weak electrostatic, dipole bonds [3]. The presence of adsorbed proteins triggers subsequent host reactions, such as blood clot formation, inflammation and cell attachment on biomaterials [4]. However, protein adsorption on biomaterials is mostly an haphazard process, which is mainly driven by the chemical and physical characteristics of the material ~~and by protein availability and conformation~~, and which may result in the adsorption of proteins which do not convey specific or useful stimuli to cells [5,6]. As a consequence the control of protein adsorption through the supply of adequate stimuli can make cell adhesion and colonisation of the biomaterial easier, and can also affect subsequent cell behaviour. To this purpose, approaches available in the literature to stimulate cell function and enhance bone formation include the use of chemical and physical treatments, which allow the surface enrichment with functional groups that preferentially bind ~~certain~~ ~~desired~~ proteins [7-9]. ~~Otherwise~~ ~~Alternatively~~, implantable biomaterials can be ~~directly~~ coated with ~~many different~~ bioactive molecules, which mimic the natural ECM [10-16], or ~~protein fragments, with e.g.~~ RGD sequences ~~and other recognition sequences for integrins~~ [17].

A possible alternative approach to enhance the biological activity of a biomaterial is to promote adsorption of bioactive molecules from the host, by means of receptors that can specifically bind to and enrich the biomaterial surface with proteins of interest, to provide an endogenous stimulus to cell colonisation. To ~~reach this goal~~ ~~this purpose~~, in the present study ~~describes~~ ~~the coating of~~ hydrogel scaffolds ~~with~~ ~~were coated with~~ ssDNA aptamers (Fig.1A). Aptamers are small, single stranded biomolecules, typically oligonucleotides, less than ~~one hundred~~ 100 residues long [18,19], which specifically bind to a target molecule

[20]. Aptamers therefore act as antibodies by binding target molecules ~~maintaining its native conformation~~, but without ~~the some of the~~ drawbacks ~~connected~~ associated to the use of antibodies, i.e. immunogenicity and low stability.

Biomaterials coating with aptamers is a less explored field in literature, but with a ~~the promising~~ potential ~~for promising results~~. Hoffmann et al. pioneered the field in 2007 by grafting aptamers against endothelial precursor cells on vascular prosthesis grafts to sort them from the bloodstream [21]. Similarly, Chen et al. in 2012 designed an artificial ECM by grafting aptamers against surface cell receptors on PEG-based hydrogels and thus dramatically improved cell adhesion to the substrate [22].

For the present report, hyaluronic acid-based hydrogels, commonly used for stem cells culture, were functionalized with aptamers against Fibronectin. Fibronectin was chosen as a target for the present study report because it is a widely available protein in plasma, so it would readily be available after implant insertion, in an hypothetical surgical scenario. Moreover Fibronectin plays a pivotal role in wound healing by forming ~~providing~~ a substrate for cell attachment during the formation of granulation tissue ~~formation~~ [23], and ~~for because of~~ that ~~reason~~ it has been proposed as a coating for implantable biomaterials [24-26]. Although the use of anti-Fibronectin aptamers hasve been previously shown to serve as a tool to inhibit cell adhesion by impeding the interaction of integrins with cell binding domains [27], this anti-Fibronectin aptamers has been proposed to inhibit cell adhesion by impeding the interaction of integrins with cell binding domains [27], this is the first report of aptamers directed against Fibronectin to improve cell adhesion on a scaffold. Our work shows that aptamers against proteins with adhesive properties such as Fibronectin can increase scaffold colonisation by cells, enhancing their adhesion and growth both in 2D and in 3D cultures *in vitro*.

## 2. Materials and Methods

**2.1 Aptamer specificity for Fibronectin** - We had ssDNA aptamers screened against bovine Fibronectin (ATW008 Fibronectin aptamer, Base Pair Biotechnologies, Pearland, TX) and modified with a short carbon chain containing a S-S bond on their 3' end, and with a biotin on their 5' (IDT, Coralville, IA; USA) (Fig.1B-C). [Aptamer sequence is property of Base Pair Biotechnologies and could not be disclosed in the present manuscript.](#)

To confirm aptamer specificity for Fibronectin, a Western Blot analysis was performed. A volume of 50µg of Fetal Bovine Serum (FBS, ThermoFisher, Waltham, MA; USA) proteins were electrotransferred onto a PVDF membrane (Immuno-Blot PVDF membrane for protein blotting, BIO-RAD, Hercules, CA; USA) at 130V for 1 hour, after 10% polyacrylamide gel (Acrylamide/Bis-Acrylamide 30%, Sigma-Aldrich) 1D electrophoresis separation in no denaturing conditions at 100V for 1 hour. After blocking, membrane was incubated with 10ml of aptamer 2µg/ml in 5% Bovine Serum Albumine (BSA, Sigma-Aldrich) at 4°C overnight. An anti-biotin peroxidase-conjugated antibody (A0185, Sigma-Aldrich) diluted 1:40000 was used as secondary to detect the presence of aptamer on the membrane. Blot was finally developed using the luminol (Clarity™ Western ECL Substrate, BIO-RAD). Immunoreactive bands were visualized by a 5 minutes exposure (Kodak X-OMAT, Milan, IT).

**2.2 Aptamer preparation** - To use aptamers for hydrogels functionalization, the S-S bonds at the 3' ends were reduced with a 2mM solution of Tris(2-carboxyethyl)phosphine hydrochloride(TCEP, Sigma-Aldrich) pH 7.8 for 2 hours prior to use, so that they yielded free thiol-groups, freshly before use. TCEP-treated aptamers were purified on a chromatographic column (mini Quick Spin Oligo Columns, Roche Life Science, Branford, CT; USA) to remove TCEP before addition to hydrogel, following manufacturer's recommendation.

**2.3 Scaffold preparation** - We selected a 3D matrix which could be easily enriched with aptamers, such as Hyaluronic acid/Polyethyleneglycole-based hydrogels (tHA/PEGDA) (HyStem™ Hydrogel Kit, ESI-BIO, Biotime Inc., San Francisco, CA; USA). These hydrogels possess functional groups that can be used to easily bind organic molecules such as aptamers and are often used to culture stem cells, because they offer scant adhesion to cells.

Reduced aptamers were mixed to PEGDA at the final concentration of 14µM and incubated for 20 minutes prior to the addition of tHA in a 1:1 ratio with PEGDA. Gels were allowed to set for 1 hour. The thiol groups on the 3' end of aptamers bound to PEGDA acrylate groups through a nucleophilic addition (Michael's addition) and thus immobilized aptamers on the matrix.

**2.4 Validation of hydrogels functionalization** – The presence of aptamers on hydrogels was confirmed with an UV-fluorescence DNA intercalating dye (SYBR Safe DNA gel stain, Life Technologies, Carlsbad, CA; USA). Hydrogels were prepared with three different aptamer doses, respectively with 0.5µg, 1µg and 5µg. Hydrogels were rinsed with Phosphate Buffer Saline (PBS, Sigma-Aldrich), in order to remove unbound aptamer, and subsequently covered with a 1:10000 solution of the dye, which labelled the aptamer revealing its presence under UV.

After the staining, SYBR Safe solution was removed and substituted with fresh PBS to investigate aptamer release from the functionalized scaffolds. Samples were incubated at 37°C and 5% CO<sub>2</sub>, and aliquots of PBS were collected immediately after the incubation and after 24, 48 and 144 hours. Aptamer release was evaluated through its detection in PBS aliquots with the Nanophotometer (Implen, Munich, D) and confirmed with a DNA-electrophoresis on a 0.8% agarose gel in TBA buffer (SeaKem, Lonza, Visp, CH) at 80V.

**2.5 Hydrogels selectivity binding activity for Fibronectin** – To highlight the absorption of Fibronectin on hydrogels, Bradford assay was performed. Hydrogel samples were prepared with 5µg of aptamer. Hydrogels were washed with PBS and subsequently incubated with human plasma-derived Fibronectin (Sigma Aldrich) at different concentrations, respectively 35µg/ml and 70µg/ml for 48 hours at 4°C. Aliquots of supernatants were collected after 0, 1, 2 and 48 hours and Fibronectin concentration in supernatants measured with the Bradford (BIO-RAD Protein Assay, BIO-RAD).

Moreover at 48 hours, supernatant was removed from samples, hydrogels rinsed in PBS and incubated with Bradford solution, in order to detect the amount of Fibronectin bound to the functionalized hydrogels.

Furthermore, to highlight Fibronectin on gels, staining was performed on each sample with a rabbit polyclonal anti-Fibronectin antibody (F3648, Sigma-Aldrich) for 1 hour followed by three rinses with PBS. Samples were then labelled with a FITC-anti-rabbit IgG antibody (ab97048 Abcam, Cambridge, UK). Samples were examined using a Nikon Eclipse 90i microscope (Nikon, Tokyo, Japan) equipped for fluorescence analysis. Areas covered with Fibronectin were then quantitated with the Image J (ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA).

**2.6 Cell cultures** - HOB cells were obtained from the American Type Culture Collection (LGC Standards S.R.L., Sesto S.Giovanni, MI, Italy). They were grown in Dulbecco MEM (DMEM, PAA, GE Healthcare, Uppsala, Sweden) additioned with 10% FBS, ThermoFisher) and 1% Penicillin and Streptomycin (Penstrep, Sigma-Aldrich).

Cell assays were performed in 96-well plates or in 8-well chamber slides (Nunc, ThermoFisher,) in complete medium.

**2.7 2D cultures** - For cell imaging, cells were seeded on hydrogels at the concentration of 5000 cells/well in complete medium. Cell growth was monitored on hydrogels functionalized with 0.5, 1 and 2 $\mu$ g of aptamer through the use of an inverted microscope (Leica, Wetzlar, D), and after 48 hours, areas covered with cells were quantitated through the ImageJ. For longer experimental points, only samples prepared with 2 $\mu$ g of aptamer were considered. After 96 hours of culture cells were stained with Calcein AM, while after 10 days of culture by immunofluorescence. For Calcein AM staining, culturing medium was removed and replaced with a 2 $\mu$ M PBS solution of Calcein AM (Calcein AM Solution, Sigma-Aldrich). Samples were incubated at 37°C and ppCO<sub>2</sub> 5% for 15 minutes and subsequently observed at the stereomicroscope (SMZ25, Nikon), in order to detect the entity of cell migration inside the hydrogels.

For the immunofluorescence, cells were fixed with 4% PFA for 10 minutes followed by three rinses with PBS. Cells were then permeabilized with a 0.1% aqueous solution Triton-X100 (Sigma-Aldrich) for 5 minutes followed by three rinses with PBS. Non-specific binding sites were blocked by incubating the samples in a 1% PBS solution of bovine serum albumin (BSA, Sigma-Aldrich) for 20 minutes. Cells were stained with a FITC-conjugated rabbit monoclonal anti-vinculin antibody (FAK100, Chemicon, Temecula, CA, USA) and TRITC-conjugated phalloidin (FAK100, Chemicon) for 1 hour, followed by three rinses with PBS. Nuclear counterstaining was performed by incubation with DAPI (D1306, Molecular Probes, Life Technologies) for 5 minutes followed by three rinses with PBS. All the steps were carried out inside the culture well at room temperature and in dark conditions. Treated hydrogels were then transferred to microscope slides and mounted under glass cover slips using an antifade-mounting medium (P7481, Molecular Probes, Life Technologies) for photo bleaching reduction. Samples were examined using the microscope equipped for fluorescence analysis.



After 10 days samples were immersed in 4% paraformaldehyde (Sigma-Aldrich), dehydrated in ethanol (Sigma-Aldrich), transferred to xylene, and embedded in paraffin. Six-micrometer-thick sections were stained with Hematoxylin and Eosin, and examined using a Nikon Eclipse 80i microscope (Nikon) and the softJ software to quantitate the amount of cells in both in sections.

Moreover, to investigate aptamer influence on Fibronectin adsorption and consequently on cell proliferation, cells were plated and cultured for 24 hours in the presence or in the absence of bovine Fibronectin, and subsequently observed at the inverted microscope (Leica, Wetzlar, D).

**2.8 Viability assays** - To evaluate cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (MTT, Roche Applied Science, Penzberg; D) was performed at 48 hours and Resazurin Sodium Salt assay (Resazurin Sodium Salt, Sigma-Aldrich) at 24 and 120 hours.

For the MTT, hOB cells were seeded at the density of 5000 cells/well in complete medium. Cell growth was assessed 48 hours after seeding by MTT according to manufacturer's recommendations. Briefly, 5  $\mu$ l of MTT labelling reagent (final concentration 0.5mg/ml) were added to the samples. After 4 hours of incubation at 37°C and 5% CO<sub>2</sub>, 100  $\mu$ l of solubilization solution were added and samples incubated overnight at 37°C and 5% CO<sub>2</sub>. Absorbance was finally read at 570nm by a Multiskan FC Microplate Photometer (ThermoFisher).

Similarly for the Resazurin Sodium Salt assay, hOB cells were seeded at the density of 5000 cells/well and cell metabolic activity was measured 24 and 120 hours after seeding. At each time point 20 $\mu$ l of the Resazurin Sodium Salt stock solution (final concentration 0.15mg/ml) were added every 100 $\mu$ l of culturing medium and samples were incubated at 37°C and 5% CO<sub>2</sub> for 4 hours. Fluorescence was finally excited at 560nm and emitted at

585nm by a Multiskan Ascent microcell plate reader (Thermo LabSystems, Helsinki, Finland).

**2.9 3D cultures** - For cell encapsulation, hOB cells were resuspended in complete medium, and mixed to tHA. PEGDA with or without aptamers was added to the cell mixture and incubated for 1 hour to allow for the gels to set. Gel cylinders were then placed in a 8-well chamber slide and cultured as described above. After 10 days, gels were fixed with 4% PFA for 10 minutes followed by three rinses with PBS. Specimens were dehydrated through ethanol series at 4°C, and then embedded in Paraffin and sectioned. Sections were then stained with Hematoxylin-Eosin and observed at optical microscopy (Axiscope, Zeiss, Oberkochen, D). Alternatively, sections were stained with a FITC-conjugated rabbit monoclonal anti-vinculin antibody (FAK100, Chemicon), with TRITC-conjugated phalloidin (FAK100, Chemicon) and nuclear counterstaining was performed by incubation with DAPI (D1306, Molecular Probes, LifeTechnologies), as described for the immunofluorescence in paragraph 2.7. Samples were examined using a microscope equipped for fluorescence analysis.

**2.10 Statistical analysis.** Data were analyzed using Prism 6 (GraphPad, La Jolla, CA, USA). All values are reported as the mean  $\pm$  Standard Deviation of three repeated experiments. Differences between group means were evaluated with either *t* Test, one-way ANOVA, or two-way ANOVA statistical tests with Tukey post-test and differences were considered significant when  $p < 0.05$ .

### **3. Results**

**3.1 Aptamer specificity for Fibronectin** - Aptamers were tested for binding specificity to Fibronectin through Western Blot assay. Two main bands were visible, with weights

compatible with Fibronectin whole protein and sub-units (Fig. S1). Moreover, a third, fainter band of approximately 120 kDa was observed.

**3.2 Validation of hydrogels functionalization** – To detect the presence of aptamers on hydrogels, ~~hydrogels~~ were functionalized with different doses of aptamers, and their presence was marked with an UV-fluorescence DNA intercalating dye. Moreover, aptamer release was evaluated through the nanophotometer by measuring aptamer concentration in fresh PBS. We observed (Fig.2A-B) an increase in DNA labelling on hydrogels with increasing aptamer concentrations, indicating that the scaffolds bound the aptamers, with very high efficiency regardless of dosage (Fig.2C). Interestingly, ~~we~~ spectrophotometry ~~was~~ not able to detect the presence of DNA in supernatant ~~by spectrophotometry~~, suggesting a very low release rate from hydrogels (Fig.2C). These results were confirmed by DNA electrophoresis which did not reveal any DNA-residual DNA in solution (Fig.2D).

**3.3 Aptamers enrich scaffolds for human Fibronectin** - To investigate whether scaffolds functionalized with aptamers could be enriched for the target protein Fibronectin, hydrogels were ~~prepared~~ added with 5µg of aptamer and incubated with concentrations of human Fibronectin comparable to those observed in culture conditions, respectively 35 and 70µg/ml. The Bradford assay showed a more marked decrease in Fibronectin in supernatants when aptamers were present as compared to control hydrogels (Fig.3A-D) ~~for~~ both concentration groups of Fibronectin. Consistently with this, the Bradford assay also revealed a higher amount of Fibronectin on aptamer-enriched hydrogels as compared to control substrates (Fig.3C-D).

The presence of Fibronectin was also investigated by immunofluorescence, using an anti-Fibronectin antibody (Fig.3E-F). Addition of aptamers greatly enhanced Fibronectin adsorption on hydrogels, as indicated by a greater degree of fluorescence, which was then

quantitated as reprinted in Figure 3G: consistently with previous data, fluorescence was ~~shown to be~~ significantly higher in the presence of aptamer than on the control.

### 3.4 Cell adhesion on hydrogel matrices is enhanced by anti-Fibronectin aptamers -

To investigate the hypothesis that aptamers-enriched hydrogels could promote cell adhesion, hOB cells were ~~plated~~seeded on the surface of hydrogels in the presence or in the absence of aptamers. Cells appeared viable in both the groups throughout the whole experiment. However, although few hOB cells were observed on control hydrogels after rinsing (Fig.4A), suggesting poor cell attachment, many cells ~~had~~ attached and proliferated on aptamer-enriched gels, creating little cell clusters that covered the whole surface of the well (Fig.4B). Furthermore, the amount of hydrogel ~~s~~ area covered by cells in the presence or in the absence of aptamers, was quantitated and plotted in Figure 4C.

The area covered with cells was significantly higher on aptamer-coated scaffolds and is resulted to be dependent on the concentration of aptamer used for the functionalization (Fig.S2). When cells were seeded on the hydrogels in the absence of serum, the effect of aptamer on cell attachment was severely impaired (Fig. S3), indicating that serum proteins are required for aptamer activity on cells.

Similarly, sections of samples embedded in paraffin after 10 days of culture showed ed more numerous cells in the presence of aptamers, suggesting a favourable environment for osteoblasts (Fig. 4D-E). The amount of gel colonized by cells was quantitated and plotted in Figure 4F.

Moreover, we quantitated cell viability with the MTT assay after 48 hours of culture (Fig.4I), and with the Resazurin Sodium Salt assay after 24 and 120 hours (Fig.4L). DataThes collected data confirmed that aptamers enhance cell viability and proliferation, showing significant differences just after 48 hours.

Cells cultured as described above were also fixed and stained with anti-Vinculin antibody, TRITC-Phalloidin and DAPI for fluorescent labelling of focal adhesions, microfilament and nuclei respectively. More cells were observed on aptamer-enriched hydrogels and their cytoplasm appeared more spread and richer in adhesion complexes (Fig.4G-H). Significantly more cells were observed on PEGDA-tHA hydrogels after addition of anti-Fibronectin aptamers.

**3.5 Cell colonisation of 3D matrices is enhanced by anti-Fibronectin aptamers** - We proceeded to investigate whether aptamers could promote cell growth in 3D hydrogel models. To this purpose, hOB cells were encapsulated in PEGDA-tHA hydrogels in the presence or in the absence of anti-Fibronectin aptamers. After 10 days, cells were present in both groups, as they had been encapsulated in the scaffold and thus more easily retained. However, beside cells were more numerous in the presence of aptamers (Fig.5B), average cell morphology appeared quite different: most cells were very elongated in the control group and possessed long, sometimes with neurite-like appearance, cytoplasmic extroflexions (Fig.5A). In contrast, cells in aptamer-containing gels had wider cytoplasm with broad podosomes (Fig.5B). Fluorescence observation confirmed the presence of a higher number of cells in the presence of aptamers (Fig.5C-D).

**3.6 Aptamers promote cells migration into the hydrogels** - ~~The entity of o~~steoblasts migration into the gels in the presence or absence of aptamers was analyzed after 96 hours through ~~the~~ staining with the Calcein AM. Cells were obsved were present on multiple focus planes in the presence of aptamers (Fig.6 A-D). Moreover, cell clusters in control hydrogels were smaller than in the presence of aptamers (Fig.6 A,B,E).

## 4. Discussion

The ECM is physiologically loaded with signalling molecules, extracellular matrix proteins and growth factors, which provide cells with a vast array of signals for cell attachment, replication and differentiation. It has been shown that sequestered factors evoke a radically different biological response from growth factors merely added to a biological system, because of their different kinetics and clearance [287]. Biomaterials implanted into a tissue have a difficult goal to achieve, to integrate in such a complex environment and provide cells with a substrate ~~which that~~ should ~~as closely as possible~~ mimic their native niche as closely as possible, to promote the attachment and scaffold colonisation by cell precursors, which will ultimately differentiate along the desired lineages and mediate tissue regeneration. Biomaterials can hardly exert any desired biological effect without adsorbing proteins on their surface. Although materials tend to spontaneously adsorb proteins when they are inserted into biological tissues, this process is scarcely predictable, as it is mostly driven by protein availability and weak bonds established between protein species and material surface (Figure 1A). Hitherto, the commonest approach to control protein ~~coating adsorption~~ on biomedical devices has been to ~~enrich pre-coat them~~ enrich their surface with exogenous signalling molecules or fragments thereof [29, 30]. This has significant limits in terms of molecule availability, compatibility and cost. ~~The~~ On the other hand, the use of tissue derivatives that contain active ECM components to promote tissue regeneration, such as collagen, is often marred by complications [31], and these ~~usually which then~~ requires extensive processing to improve ~~material~~ their compatibility. Moreover, the addition of peptides such as RGD sequences to ~~the~~ biomaterials ~~may~~ provides an adequate ~~adhesion~~ substrate to ingrowing cells, as ECM does, but does not ~~address confer the need for~~ the ~~matrix~~ biomaterial ~~the capability to be able~~ to incorporate further matrix components, as they become available from growing and actively synthesising cells. An alternative approach to selectively attract and retain desired

proteins on biomaterial surfaces is to coat the ~~m-implantable surface~~ with elements able to dock specific target peptides that can be recruited from the surrounding microenvironment. Aptamers can serve for such a purpose (Figure 1B). Aptamers are oligonucleotides, whose primary and secondary structure enables them to recognise and bind specific target molecules [18], without eliciting adverse responses and without being recognised by the host's immune system, because of their size and chemical nature [32]. Conventionally, aptamers are selected using the SELEX process (Systematic Evaluation of Ligands by Exponential Enrichment) [33], which ensures absence of variability in the end-product, in contrast to antibodies, ~~that~~ which have an inherent biological variability because of the way they are developed and purified [34]. Aptamers are also more stable to degradation than antibodies and bind to their respective ligands under conditions not possible with antibodies [35].

In the present study PEGDA/tHA hydrogels were functionalised with anti-Fibronectin aptamers to enhance scaffold colonisation by cells. PEGDA/tHA hydrogels were chosen because they offer scant attachment to cells, as our results confirmed, and their chemistry allows for their easy functionalisation ~~through thanks to the availability of~~ free available acrylate groups. Our results consistently showed ed that anti-Fibronectin aptamers were stably bound to hydrogels, and no release was detected under culture conditions. Aptamers enriched hydrogels for Fibronectin, ~~an event that occurred~~ quite rapidly after medium addition (Fig. 3A-D). Fibronectin retention in turn promoted cell attachment to the scaffold and cell proliferation in a concentration-dependent fashion. Cells were grown on hydrogel scaffolds both as 2D or 3D cultures. When cultured on the ~~gel~~ surface of the gels, few cells were visible in the control group, as most ~~cells detached were rinsed away~~ by rinsing with PBS, whereas hOBs were more numerous, formed bigger clusters (Fig. 6 A-B-E) and were observed on multiple focus planes in the presence of aptamers. The use of a Z-axis motorised stereomicroscope allowed for in depth analysis of cell distribution and

revealed that cells, albeit seeded on the surface of the hydrogel, tended to grow further down into the scaffold in the presence of aptamers, whereas remained mostly localised on the surface of control gels (Fig. 6A-D). Aptamers promoted better adhesion of cells [to the hydrogels](#), as suggested by immunofluorescence but their effect was appreciated only in the presence of serum (Fig. S3), which confirms that aptamers did not act by directly binding to cells but required the presence of serum proteins. More specifically, the present aptamers were selected against Fibronectin, for which they display high affinity with a Kd of up to  $5 \times 10^{-11}$  M [36]. When tested for specificity through Western Blot assay, bands compatible with Fibronectin whole protein and subunits were visible (Fig. S1). Moreover, a third, approximately 120 kDa heavy band was also observed. Although this peptide [has not been identified yet](#), it is possible to speculate that it may ~~be represented by~~ [correspond to](#) the 120 kDa [internal cell-binding](#) fragment of Fibronectin, ~~possibly for spontaneous proteolysis by serum enzymes.~~ [Previous works have shown that digestion of Fibronectin by Metalloproteinase-9 yields a 120 kDa fragment that contains cell-binding domains \[37\]. Though it is possible that spontaneous proteolysis of Fibronectin may have occurred in vitro, no evidence to this regard was found in the literature, and binding of a different, unrelated peptide cannot be ruled out.](#) Future studies will have to ascertain the nature of this additional [molecule band](#), as, at the present time, available data do not allow to rule out a role of this ~~further~~ peptide in contributing to cell adhesion [beside Fibronectin](#).

When encapsulated, the difference in cell number between the groups was not as high as with 2D cultures (Fig. 5A-D), as cells are more easily retained in control hydrogels. However, cell shape was different: cells were elongated and projected numerous long neurite-like extroflexions in the absence of aptamers. This morphology may possibly suggest that cells were trying to increase their adhesion in control substrates to a higher degree than in aptamer-enriched gels, where cell morphology was rather quadrangular (Fig. 5B-D). In conclusion, our data support the idea that anti-Fibronectin aptamers



improve cell adhesion to a hydrogel scaffold, such as could be used in a tissue defect. The use of aptamers to improve cell colonization on scaffolds poses however some significant challenges: The binding site between aptamers and their target is important, as anti-Fibronectin aptamers have been previously shown to serve as a tool to inhibit cell adhesion by impeding the interaction of integrins with cell binding domains [27]. Our aptamers did not hinder Fibronectin interactions with cells, and did promote cell attachment on the scaffold. Exact molecular interactions could not be investigated, but cellular binding sites were likely to be left available, if cell adhesion was improved. Moreover, the choice of the target protein appears ~~however~~ to be critical, as novel properties can be conferred to medical devices by tuning the adsorbed protein species. It has been shown for instance that biomedical devices coated with aptamers targeting integrins on cell membranes can specifically sort cells out of the circulation [21] (~~Hoffmann, J.; Paul, A.; Harwardt, M.; Groll, J.; Reeswinkel, T.; Klee, D.; Moeller, M.; Fischer, H.; Walker, T.; Greiner, T.; Ziemer, G.; Wendel, H. P. 2008~~). Potentially, other bioactive molecules that are found in wounds could be targeted, such as PDGF, which is stored in platelet granules, to confer the scaffold with more specific biological effects, and that could be tuned according to the specific tissue to regenerate. The safety of this approach in living systems has yet to be determined, as the effects of released aptamers upon scaffold degradation are substantially unknown and undesired effects cannot be ruled out at this stage. Furthermore, extensive *in vivo* testing must be performed to investigate whether these aptamers can bind their target in the clinical conditions of the surgical wound, which can substantially differ from *in vitro* culture systems.

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## 6. References

- [1] Vogler EA. Protein adsorption in three dimensions. *Biomaterials* 2012;33:1201-37.
- [2] Micksch T, Liebelt N, Scharnweber D, Schwenzer B. Investigation of the peptide adsorption on ZrO<sub>2</sub>, TiZr, and TiO<sub>2</sub> surfaces as a method for surface modification. *ACS applied materials & interfaces* 2014;6:7408-16.
- [3] Kastantin M, Langdon BB, Schwartz DK. A bottom-up approach to understanding protein layer formation at solid-liquid interfaces. *Advances in colloid and interface science* 2014;207:240-52.
- [4] Hiraguchi Y, Nagahashi K, Shibayama T, Hayashi T, Yano TA, Kushihiro K, Takai M. Effect of the distribution of adsorbed proteins on cellular adhesion behaviors using surfaces of nanoscale phase-reversed amphiphilic block copolymers. *Acta biomaterialia* 2014;10:2988-95.
- [5] Clause KC, Barker TH. Extracellular matrix signaling in morphogenesis and repair. *Current opinion in biotechnology* 2013;24:830-3.
- [6] Morris AH, Kyriakides TR. Matricellular proteins and biomaterials. *Matrix biology : journal of the International Society for Matrix Biology* 2014;37:183-91.
- [7] Tegoulia VA, Rao W, Kalambur AT, Rabolt JF, Cooper SL. Surface Properties, Fibrinogen Adsorption, and Cellular Interactions of a Novel Phosphorylcholine-Containing Self-Assembled Monolayer on Gold. *Langmuir* 2001;17:4396-404.
- [8] Martins MC, Ratner BD, Barbosa MA. Protein adsorption on mixtures of hydroxyl- and methyl-terminated alkanethiols self-assembled monolayers. *Journal of biomedical materials research Part A* 2003;67:158-71.
- [9] Agashe M, Raut V, Stuart SJ, Latour RA. Molecular simulation to characterize the adsorption behavior of a fibrinogen gamma-chain fragment. *Langmuir : the ACS journal of surfaces and colloids* 2005;21:1103-17.
- [10] Decker JF, Lee J, Cortella CA, Polimeni G, Rohrer MD, Wozney JM, Hall J, Susin C, Wikesjo UME. Evaluation of implants coated with recombinant human bone morphogenetic protein-2 and vacuum-dried using the critical-size supraalveolar peri-implant defect model in dogs. *Journal of periodontology* 2010;81:1839-49.
- [11] Forster Y, Rentsch C, Schneiders W, Bernhardt R, Simon JC, Worch H, Rammelt S. Surface modification of implants in long bone. *Biomatter* 2012;2:149-57.

- [12] Hu X, Neoh KG, Zhang J, Kang ET, Wang W. Immobilization strategy for optimizing VEGF's concurrent bioactivity towards endothelial cells and osteoblasts on implant surfaces. *Biomaterials* 2012;33:8082-93.
- [13] Bates C, Marino V, Fazzalari NL, Bartold PM. Soft tissue attachment to titanium implants coated with growth factors. *Clinical implant dentistry and related research* 2013;15:53-63.
- [14] Leknes KN, Yang J, Qahash M, Polimeni G, Susin C, Wikesjo UM. Alveolar ridge augmentation using implants coated with recombinant human growth/differentiation factor -5 (rhGDF-5). Radiographic observations. *Clinical oral implants research* 2013;24:1185-91.
- [15] Nayak S, Dey T, Naskar D, Kundu SC. The promotion of osseointegration of titanium surfaces by coating with silk protein sericin. *Biomaterials* 2013;34:2855-64.
- [16] Mas-Moruno C, Fraioli R, Albericio F, Manero JM, Gil FJ. Novel peptide-based platform for the dual presentation of biologically active peptide motifs on biomaterials. *ACS applied materials & interfaces* 2014;6:6525-36.
- [17] Ruoslahti E. RGD and other recognition sequences for integrins. *Annual review of cell and developmental biology* 1996;12:697-715.
- [18] Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 1990;249:505-10.
- [19] Song KM, Lee S, Ban C. Aptamers and their biological applications. *Sensors* 2012;12:612-31.
- [20] Mascini M, Palchetti I, Tombelli S. Nucleic acid and peptide aptamers: fundamentals and bioanalytical aspects. *Angewandte Chemie* 2012;51:1316-32.
- [21] Hoffmann J, Paul A, Harwardt M, Groll J, Reeswinkel T, Klee D, Moeller M, Fischer H, Walker T, Greiner T, Zimmer G, Wendel HP. Immobilized DNA aptamers used as potent attractors for porcine endothelial precursor cells. *Journal of biomedical materials research Part A* 2008;84:614-21.
- [22] Chen N, Zhang Z, Soontornworajit B, Zhou J, Wang Y. Cell adhesion on an artificial extracellular matrix using aptamer-functionalized PEG hydrogels. *Biomaterials* 2012;33:1353-62.
- [23] Chatakun P, Nunez-Toldra R, Diaz Lopez EJ, Gil-Recio C, Martinez-Sarra E, Hernandez-Alfaro F, Ferrés-Padrò E, Giner-Tarrida L, Atari M. The effect of five proteins on stem cells used for osteoblast differentiation and proliferation: a current review of the literature. *Cellular and molecular life sciences : CMLS* 2014;71:113-42.
- [24] Kim S, Myung WC, Lee JS, Cha JK, Jung UW, Yang HC, Lee IS, Choi SH. The effect of fibronectin-coated implant on canine osseointegration. *Journal of periodontal & implant science* 2011;41:242-7.

- [25] Pendegrass CJ, El-Husseiny M, Blunn GW. The development of fibronectin-functionalised hydroxyapatite coatings to improve dermal fibroblast attachment in vitro. *The Journal of bone and joint surgery British volume* 2012;94:564-9.
- [26] Rapuano BE, Hackshaw KM, Schniepp HC, MacDonald DE. Effects of coating a titanium alloy with fibronectin on the expression of osteoblast gene markers in the MC3T3 osteoprogenitor cell line. *The International journal of oral & maxillofacial implants* 2012;27:1081-90.
- [27] Ogawa A, Lomita N, Kikuchi N, Sando S, Aoyama Y. Aptamer selection for the inhibition of cell adhesion with fibronectin as target. *Bioorganic Medicinal and Chemical Literature* 2004;14(15):4001-4.
- [28] Chen XD, Dusevich V, Feng JQ, Manolagas SC, Jilka RL. Extracellular matrix made by bone marrow cells facilitates expansion of marrow-derived mesenchymal progenitor cells and prevents their differentiation into osteoblasts. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 2007;22:1943-56.
- [29] Liu Y, Wu G, de Groot K. Biomimetic coatings for bone tissue engineering of critical-sized defects. *Journal of the Royal Society, Interface / the Royal Society* 2010;7 Suppl 5:S631-47.
- [30] Trajkovski B, Petersen A, Strube P, Mehta M, Duda GN. Intra-operatively customized implant coating strategies for local and controlled drug delivery to bone. *Advanced drug delivery reviews* 2012;64:1142-51.
- [31] Song E, Yeon Kim S, Chun T, Byun HJ, Lee YM. Collagen scaffolds derived from a marine source and their biocompatibility. *Biomaterials* 2006;27:2951-61.
- [32] Keefe AD, Pai S, Ellington A. Aptamers as therapeutics. *Nature reviews Drug discovery* 2010;9:537-50.
- [33] Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. *Nature* 1990;346:818-22.
- [34] Gold L, Ayers D, Bertino J, Bock C, Bock A, Brody EN, Carter J, Dalby AB, Eaton BE, Fitzwater T, Flather D, Forbes A, Foreman T, Fowler C, Gawande B, Goss M, Gunn M, Gupta S, Halladay D, Heil J, Heilig J, Hicke B, Husar G, Janjic N, Jarvis T, Jennings S, Katilius E, Keeney TR, Kim N, Koch TH, Kraemer S, Kroiss L, Le N, Levine D, Lindsey W, Lollo B, Mayfield W, Mehan M, Mehler R, Nelson SK, Nelson M, Nieuwlandt D, Nikrad M, Ochsner U, Ostroff RM, Otis M, Parker T, Pietrasiewicz S, Resnicow DI, Rohloff J, Sanders G, Sattin S, Schneider D, Singer B, Stanton M, Sterkel A, Stewart A, Stratford S, Vaught JD, Vrkljan M, Walker JJ, Watrobka M, Waugh S, Weiss A, Wilcox SK, Wolfson A, Wolk SK, Zhang C, Zichi D. Aptamer-based multiplexed proteomic technology for biomarker discovery. *PloS one* 2010;5:e15004.
- [35] Jayasena SD. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clinical chemistry* 1999;45:1628-50.

- [36] Aptamer Information, Fibronectin aptamer ATW0008 <http://www.basepairbio.com/wp-content/uploads/2013/05/Fibronectin-ATW0008-603-datasheet.pdf>. Base Pair Biotechnologies.
- [37] Al-Hazmi N, Thomas GJ, Speight PM, Whawell SA. The 120kDa cel-binding fragment of fibronectin up-regulates migration of alphabeta6-expressing cells by increasing matrix metalloproteinase-2 and -9 secretion. *European Journal of Oral Science* 2007;115(6):454-8.

## 7. FIGURE LEGENDS

**Figure 1** (A) Diagram representing the rationale for aptamer-coated scaffold to retain specific target proteins. Un-coated scaffolds adsorb proteins from the extracellular environment based on their availability and their chemistry, mostly due to aspecific weak bonds (Control). Aptamers specifically retain target proteins and enrich scaffolds for the desired biological stimulus (Functionalized Scaffold). (B) 3D rendering and (C) 2D reconstruction of anti-Fibronectin aptamer (Courtesy of Dr. Rafal Drabek).

**Figure 2** Aptamer binding to hydrogels. (A) Hydrogels containing increasing concentration of anti-Fibronectin aptamers were labelled with an intercalating dye, an increase in fluorescence with increasing aptamer concentrations was observed and quantitated through ImageJ. (B) Correlation between the amount of aptamer used for the functionalization and fluorescence was quantified through the ImageJ. (C) To investigate whether aptamers were released from the hydrogels, aptamers were quantitated in supernatants by spectrophotometry. (D) Supernatants were furthermore analyzed through agarose electrophoresis, which did not reveal any DNA residual in solution: line 1, supernatant of the gel containing 0.5µg of aptamer after 0h of incubation in fresh PBS - line 2, supernatant of the gel containing 1µg of aptamer after 0h of incubation in fresh PBS - line 3, supernatant of the gel containing 5µg of aptamer after 0h of incubation in fresh PBS - line 4, supernatant of the gel containing 0.5µg of aptamer after 24h of incubation in fresh PBS - line 5, supernatant of the gel containing 1µg of aptamer after 24h of incubation in fresh PBS - line 6, supernatant of the gel containing 5µg of aptamer after 24h of incubation in fresh PBS - line 7, supernatant of the gel containing 0.5µg of aptamer after 48h of incubation in fresh PBS - line 8, supernatant of the gel containing 1µg of aptamer after 48h of incubation in fresh PBS - line 9, supernatant of the gel containing 5µg of

aptamer after 48h of incubation in fresh PBS - line 10, supernatant of the gel containing 0.5µg of aptamer after 144h of incubation in fresh PBS - line 11, supernatant of the gel containing 1µg of aptamer after 144h of incubation in fresh PBS - line 12, supernatant of the gel containing 5µg of aptamer after 144h of incubation in fresh PBS - line 13, fresh PBS - line 14, 5µg aptamer.

**Figure 3** Fibronectin binding to hydrogels. (A,B) Fibronectin adsorption on hydrogels was monitored through Bradford assay, which showed a fast decrease of Fibronectin in supernatants when aptamers were present if compared to control hydrogels in both the (A) 35 and (B) 70 µg/ml Fibronectin groups. (C,D) Consistently with this, Bradford assay also revealed a higher amount of Fibronectin adsorbed on aptamer-enriched hydrogels if compared to control substrates after 48 hours in both the (C) 35 and (D) 70 µg/ml Fibronectin groups. (E,F) To reveal the presence of Fibronectin on hydrogels with (F) or without (E) aptamers, samples were incubated for 1 hour with DMEM enriched with 10% FBS and labelled with a primary anti-Fibronectin antibody and a secondary FITC-labelled anti-rabbit IgG antibody for immunofluorescence. (G) Fluorescence was quantitated using ImageJ software. Aptamers significantly enriched hydrogels for Fibronectin.  $*=p<0.05$ .

**Figure 4** Aptamers promoted cell adhesion and proliferation in 2D cultures. (A,B) Microphotographs of human osteoblasts on hydrogels without (A) or with aptamers (B), after 48 hours of culture. (C) Quantification of tHA/PEGDA area covered by human osteoblasts, as observed in Fig. 5A,B. (D,E) Six micrometer sections of embedded hydrogels without (D) or with aptamers (E) after 10 days of culture. (F) Quantification of tHA/PEGDA area colonized from human osteoblasts, as observed in Fig. 5D,E. (G,H) Immunofluorescence observation of human osteoblast cultured on hydrogels in the absence (G) or in the presence (H) of aptamers, cells were labelled for Vinculin with a



FITC-conjugated rabbit monoclonal anti-vinculin antibody (green), for microfilaments with a TRITC-conjugated phalloidin (red) and for nucleus with DAPI (blue). (I) Cell viability on control and on hydrogel with aptamers, assessed with the MTT after 48 hours of culture.

(L) Histogram showing human osteoblasts viability/metabolic activity on hydrogels with or without aptamers 24 and 120 hours after seeding, cell viability was measured by Resazurin Sodium Salt Assay.

**Figure 5** Aptamers promote cell adhesion and proliferation in 3D cultures. (A-D) Encapsulated human osteoblasts in hydrogels in the absence (A,C) or in the presence (B,D) of aptamers after 10 days of cell culture, and after Hematoxylin-Eosin staining (A,B) or after immunofluorescence observation (C,D); for immunofluorescence cells were labelled for Vinculin with a FITC-conjugated rabbit monoclonal anti-vinculin antibody (green) and for nucleus with DAPI (blue). Cells were more numerous in the presence of aptamers (B,D) and their morphology was less elongated than in control hydrogels (A,C).

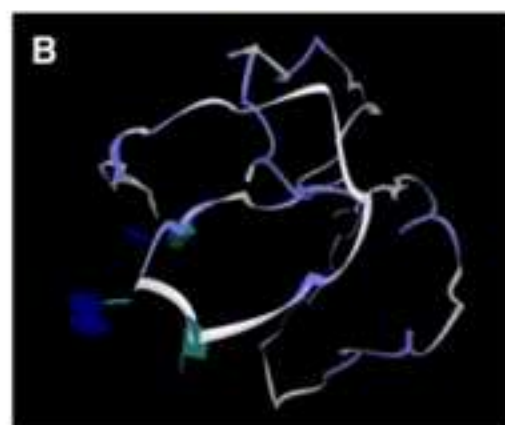
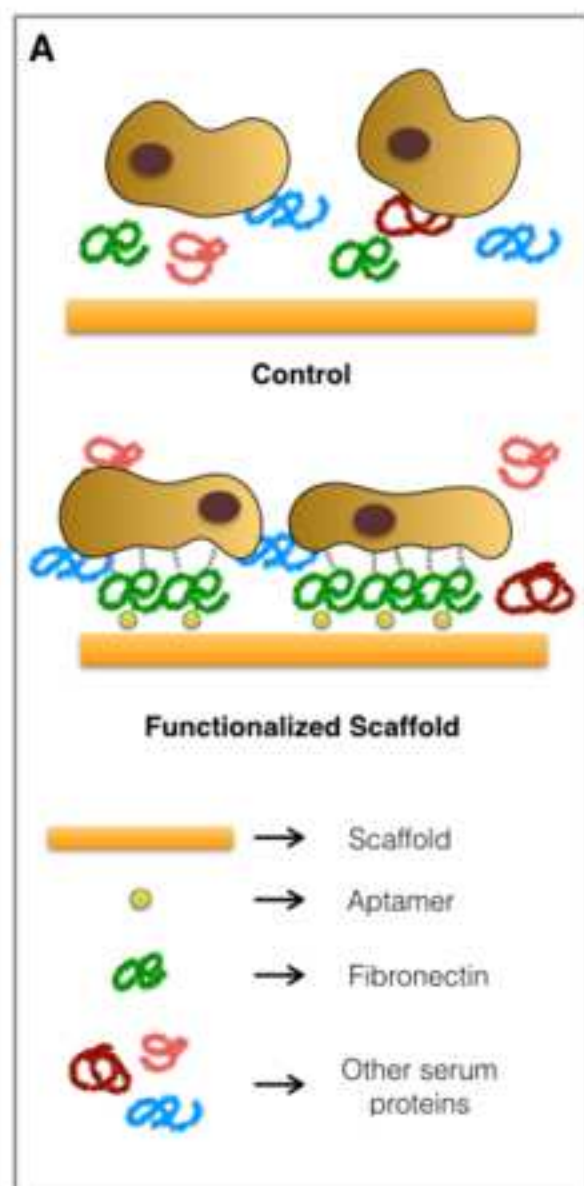
**Figure 6** Aptamers promoted cell migration into hydrogels. (A,B) Human osteoblasts stained with Calcein AM in the absence (A) or in the presence of aptamers (B). The presence of out-of-focus cells confirms the migration of cells into both the hydrogels. (C) Z-plot of cell multiple focus planes and (D) 2D graphic reconstruction of the phenomenon. (E) Cluster dimension in control and aptamer-enriched hydrogels, quantitated by ImageJ.

**Figure S1** Western Blot analysis of Fetal Bovine Serum, stained with anti-Fibronectin aptamers and revealed using an anti-biotin peroxidase-conjugated antibody diluted 1:40000.

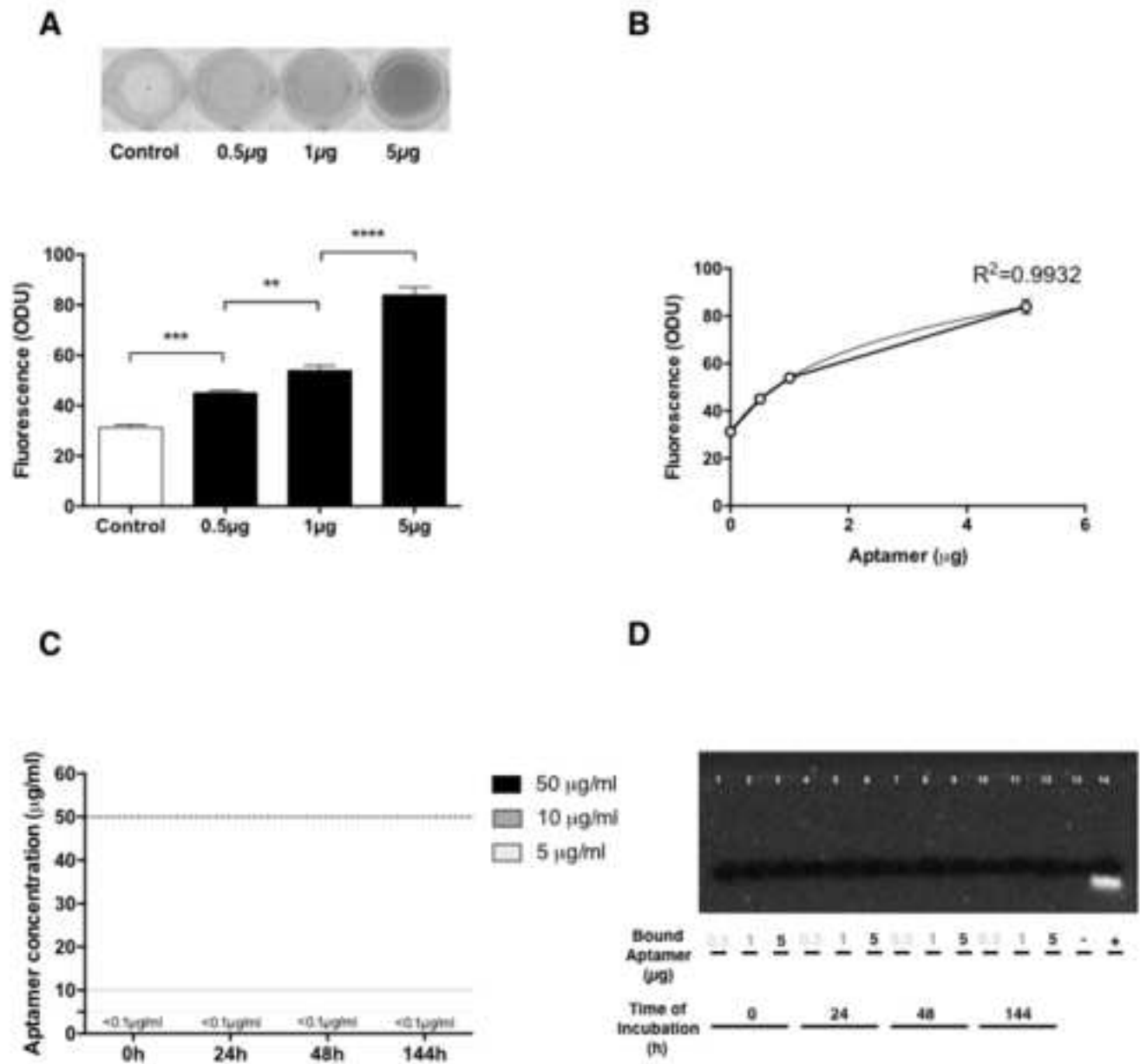
**Figure S2** (A-H) Microphotographs of human osteoblasts on hydrogels with increasing doses of aptamers, after 24 (A-D) and 48 hours (E-H) of culture. (I) ImageJ quantitation of areas covered with cells on hydrogels films with increasing doses of aptamer after 48 hours.

**Figure S3** (A-D) Microphotographs of human osteoblasts on hydrogels without (A,C) or with aptamers (B,D), in the absence (A,B) or in the presence of serum Fibronectin (C,D). The combined presence of Fibronectin and aptamers lead to Fibronectin adsorption at the surface and the consequent adhesion of osteoblasts (D).

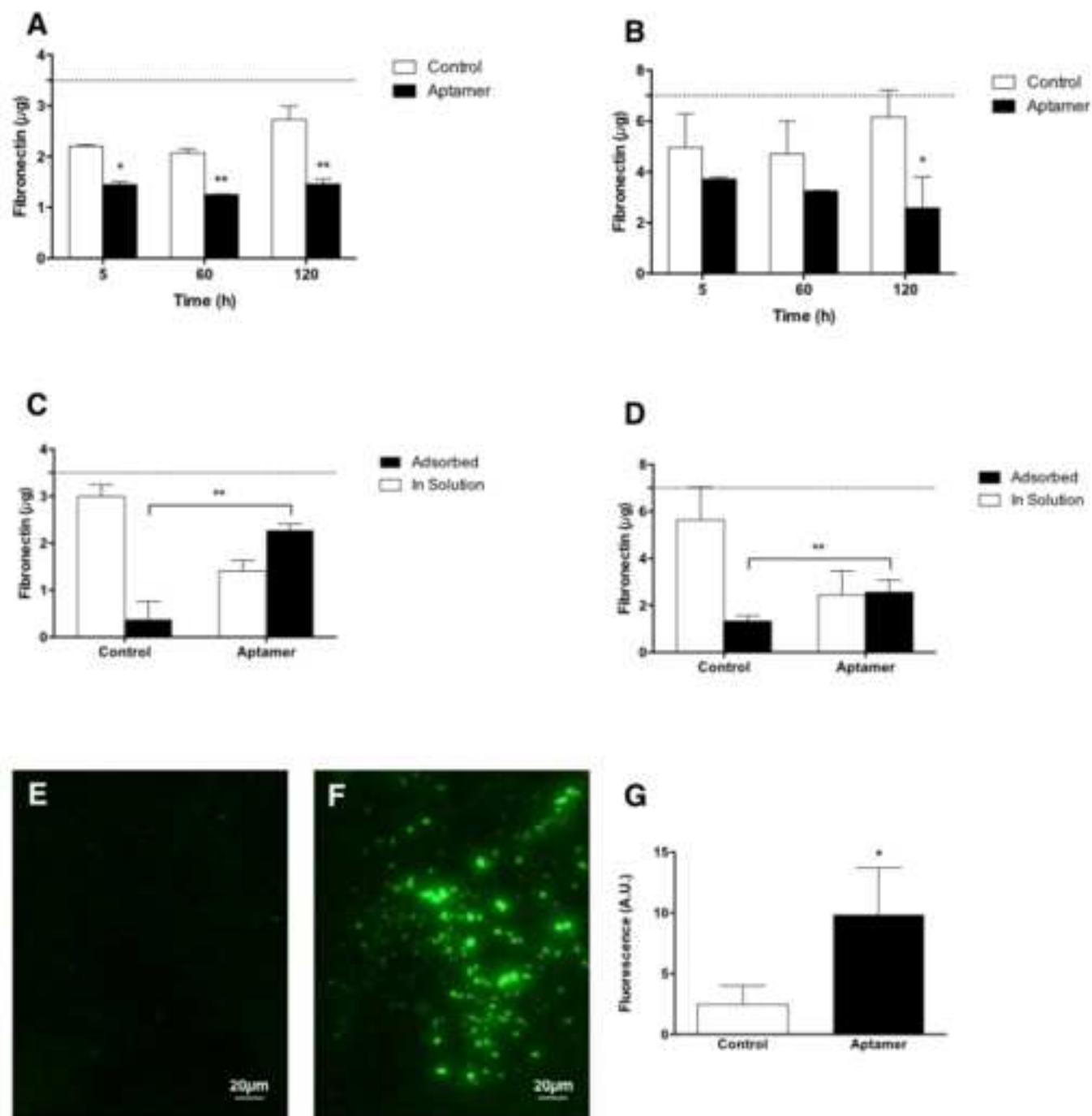
# Figure 1



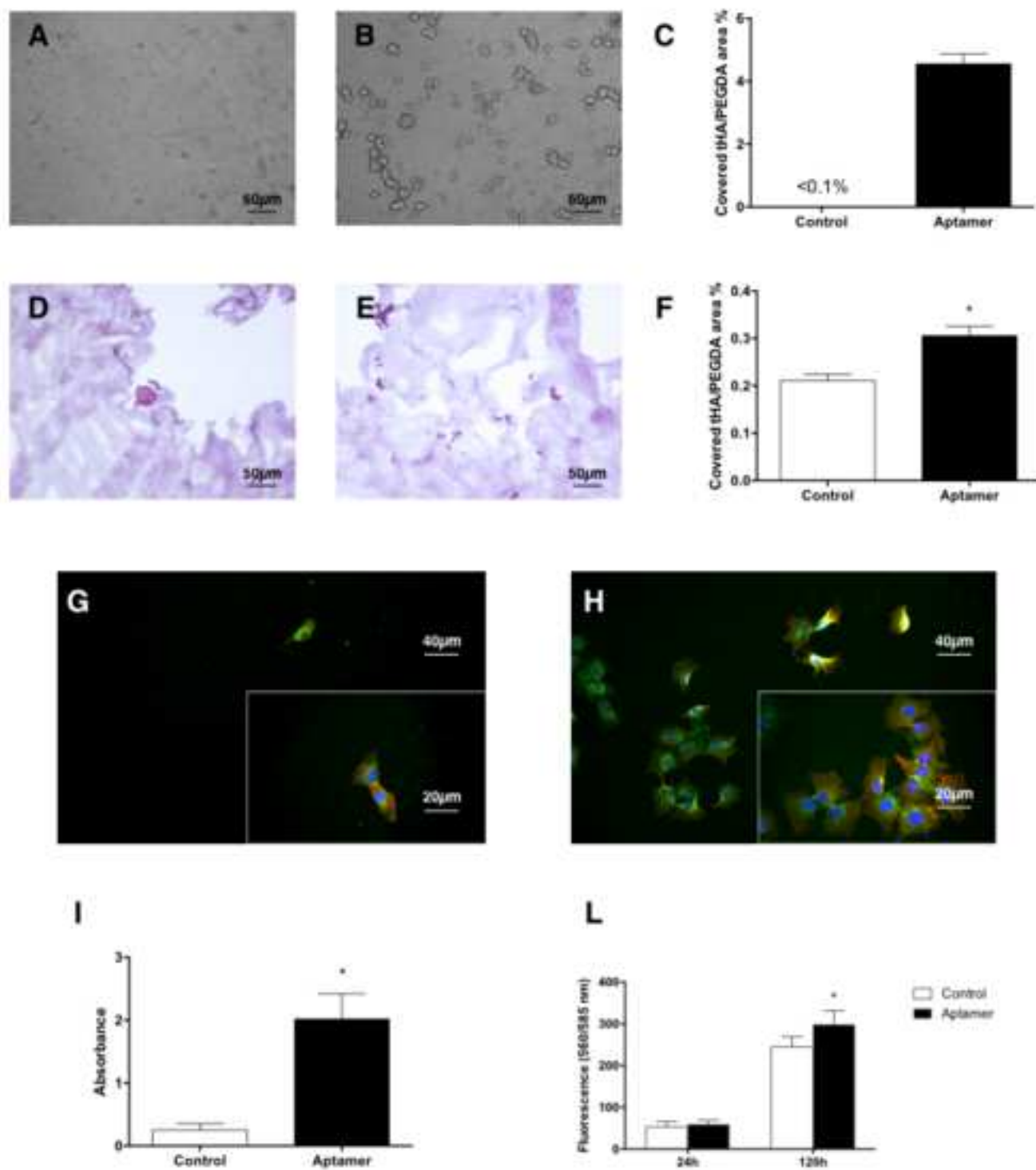
## Figure 2



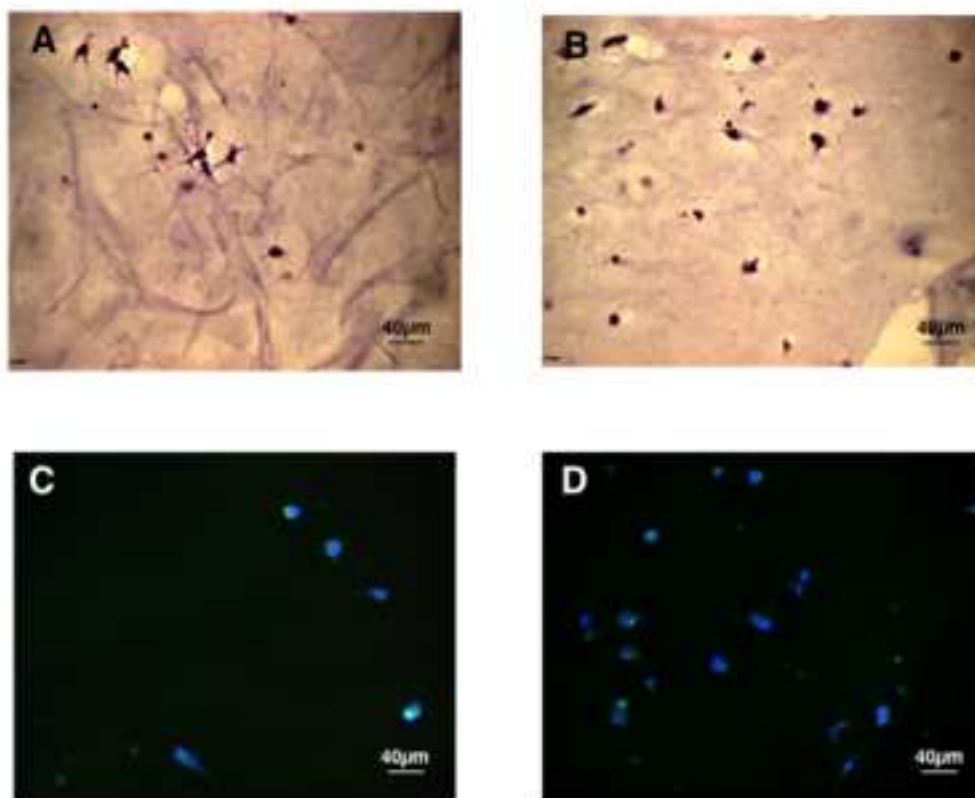
## Figure 3



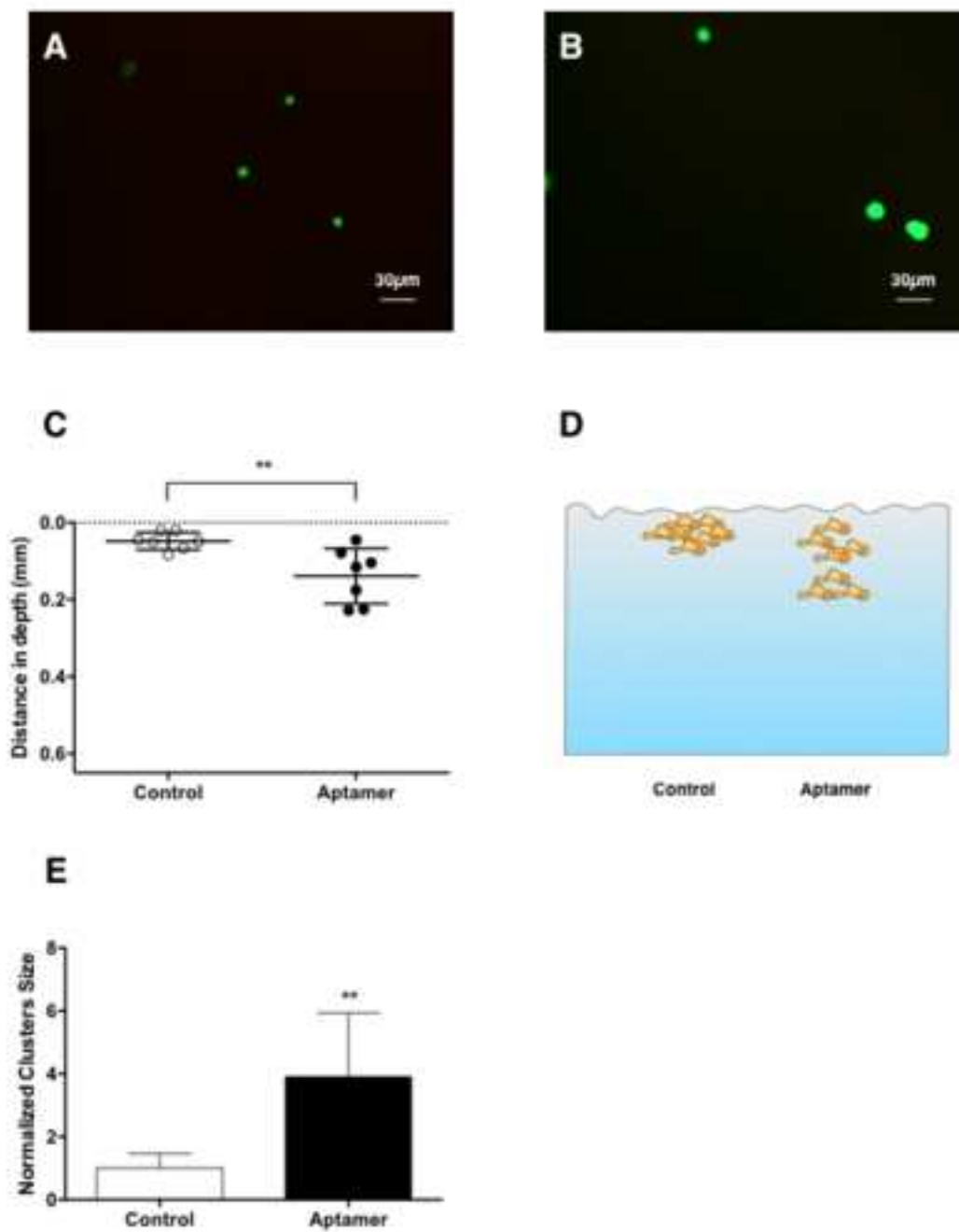
# Figure 4



## Figure 5

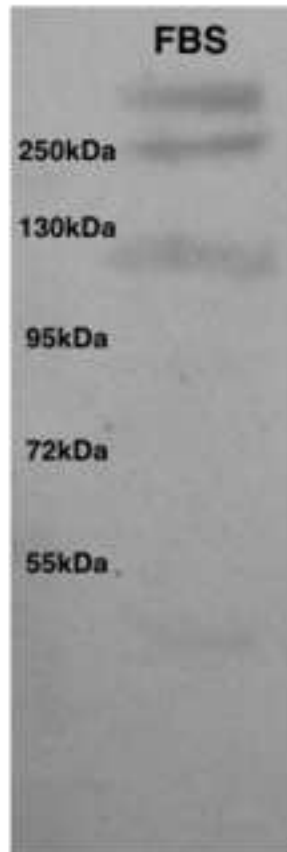


# Figure 6

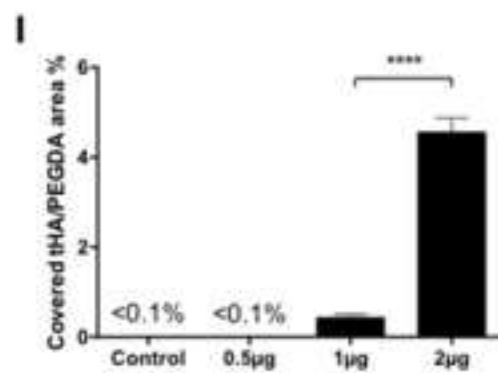
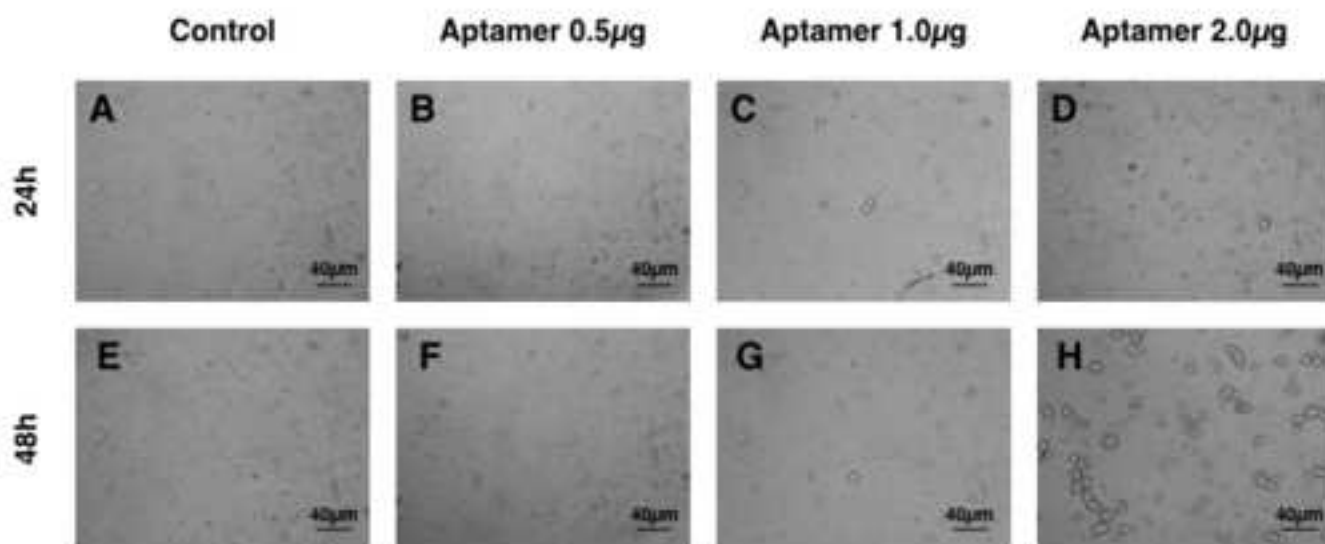




# Figure 1 Suppl



## Figure 2 Suppl



## Figure 3 Suppl

