Labeling of Endothelial Cells with Magnetic Microbeads by Angiophagy

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ABSTRACT

Objectives. Attachment of magnetic particles to cells is needed for a variety of applications but is not always possible or efficient. Simpler and more convenient methods are thus desirable. In this study, we tested the hypothesis that endothelial cells (EC) can be loaded with micron-size magnetic beads by the phagocytosis-like mechanism 'angiophagy'. To this end, human umbilical vein EC (HUVEC) were incubated with magnetic beads

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conjugated or not (control) with an anti-VEGF receptor 2 antibody, either in suspension, or in culture followed by re-suspension using trypsinization.

Results. In all conditions tested, HUVEC incubation with beads induced their uptake by angiophagy, which was confirmed by (i) increased cell granularity assessed by flow cytometry, and (ii) the presence of an F-actin rich layer around many of the intracellular beads, visualized by confocal microscopy. For confluent cultures, the average number of beads per cell was 4.4 and 4.2, with and without the presence of the anti-VEGFR2 antibody, respectively. However, the actively dividing cells took up 2.9 unconjugated beads on average, but this number increased to 5.2 if the binding was mediated by the antibody. Magnetic pulldown increased the cell density of beads-loaded cells in porous electrospun poly-capro-lactone scaffolds by a factor of 4.5 after 5 min, as compared to gravitational settling ($p < 0.0001$).

Conclusion. We demonstrated that EC can be readily loaded by angiophagy with micron-sized beads while attached in monolayer culture, then dispersed in single-cell suspensions for pulldown in porous scaffolds and for other applications.

Keywords: angiophagy; electrospun scaffold; endothelial cells; magnetic microbeads; phagocytosis; poly-capro-lactone

INTRODUCTION

Cell manipulation with attached magnetic beads has many biotechnology uses. For example, magnetic force provides a refined tool for spatial positioning and controlled stretching of cells (Lele et al. 2007), or their pulldown in porous scaffolds, a technique increasingly used in tissue engineering. Specifically, the positioning of endothelial cells (EC) within a porous scaffold has the potential to address some of the major obstacles facing the field of tissue engineering with regards to the integration of scaffolds within recipients post-implantation. A standing issue facing the incorporation of engineered scaffolds and tissues into the recipient is the commonality of reduced survival and loss of function of the contained cells. The root cause is the lack of a pre-existing vasculature system to supply them with the needed perfusion. EC have the ability to form microvascular networks which, once implanted *in vivo*, are capable of connecting to the recipient's pre-existing vasculature, alternatively or in addition to improving vascularization of the implanted construct (Joddar et al. 2017).

The ideal format of a scaffold for the implantation of engineered tissue is a porous one, as this structure allows for cellular ingrowth and thus better merging of the construct with the patient's pre-existing vasculature. In addition, a porous medium allows the invasion of fibrovascular tissue to assist with the viability of the construct's pre-existing cell population (Joddar et al. 2017). However, bringing the desired cells in optimal locations and concentrations within porous scaffolds is challenging (Villalona et al. 2010). The problem is specifically relevant for fibrillar scaffolds obtained by electrospinning, where almost all parameters other than cell incorporation can be more easily controlled (Kishan and Cosgriff-Hernandez 2017). This ability to control scaffold parameters is important, given the fact that implant-host integration and avoidance of immune response to the implant requires specific scaffold characteristics, with some studies showing a window in the tens of microns in terms of pore size for host acceptance of the implant (Joddar et al. 2017). Gravitational settling of the cells on top of the scaffold, as performed when preparing two-dimensional cultures, does not allow an effective scaffold penetration by cells (Mirensky et al. 2010), which, as discussed before, is paramount to encouraging the formation of microvasculature. The co-deposition of cells during scaffold preparation (Stankus et al. 2006), centrifugation (Godbey et al. 2004), or vacuum-assisted cell seeding (Udelsman et al. 2011) were previously proposed. All these

approaches have advantages, but also limitations concerning the fine-tuning of cells placement, as well as their post-inclusion integrity, as reviewed in (Villalona et al. 2010).

An alternative method of scaffold seeding is the use of magnetic force (Dobson 2008). Cells can be magnetically labeled either with nano-particles that bind to the cell surface (followed or not by endocytosis), or with larger beads that are phagocytosed. Magnetic nano-particles have been previously investigated for *in vivo* tagging of EC (Smith et al. 2007), for their retention on metallic stents (Pislaru et al. 2006a), or recently for disrupting the blood-brain barrier by pulling the inter-endothelial junctions (Qiu et al. 2017). Bone marrow stromal cells have been labeled with cationic liposomes containing magnetite nanoparticles, internalized by the cells while in suspension. In this case, the nanobeads-labeled cells needed to be attracted into porous hydroxyapatite scaffolds by a relatively strong magnet, for one hour (Shimizu et al. 2007). Larger particles, e.g., micron-sized commercially-available beads, would be preferable since a stronger magnetic force (proportional to particle's mass) can be generated as compared to nano-particles.

Herein, we tested the labeling of the EC with relatively larger magnetic microbeads. Building on the observation that EC in culture and *in vivo* readily take up apoptotic erythrocytes by phagocytosis [particularly those becoming more rigid (Fens et al. 2010)] and tumor cells (Fens et al. 2008), we sought to use this phagocytic mechanism to efficiently label the EC with microbeads. Endothelial phagocytosis, long known to physiologists as a function shared by EC with the 'professional phagocytes' (Wake et al. 2001; Xie et al. 2012), recently received a renewed attention (Grutzendler et al. 2014; Rengarajan et al. 2016), as well as the designated name of 'angiophagy' (Grutzendler et al. 2014). However, to our best knowledge this property has not been yet intentionally exploited for EC labeling. Here we show that, when used with cells still attached to their culture substrate followed by trypsinization, this phenomenon allows the efficient preparation of beads-containing cell suspensions with much less aggregation, one of the major drawbacks of magnetic bead labeling methods so far.

MATERIALS AND METHODS

Cells and Beads. Human umbilical vein EC (HUVEC, from ScienCell, Carlsbad, CA) were maintained in EGM-2 endothelial differentiation medium (Lonza, Morristown, NJ). Anti-biotin MACSiBead particles ~3.5 µm

in diameter (Miltenyi, Auburn, CA) were conjugated in our laboratory with a biotinylated mouse anti-human VEGFR2 antibody (Miltenyi). Cells in suspension were incubated with the beads thus prepared under gentle rotation, for 30 min at 37°C and 5% CO_2. Adherent cells, either confluent or sub-confluent, were maintained in T-25 or 6-well tissue-culture type plastic plates (Corning, Corning, NY) and were incubated with labeled beads overnight. In all cases, we maintained the beads-to-cell ratio to approximately 20:1. After incubation, excess beads were washed out, and the adhered cells were lifted by 0.5% trypsin/EDTA and counted in a hemocytometer. The beads-containing cells were preselected via a lateral magnetic pulling by loading in a 1.5 mL centrifuge tube that was placed in a MagnaSep magnetic separator (Invitrogen, Waltham, MA) for 5 min. The supernatant was removed and the retained cells were re-suspended in fresh culture medium and used for the experiments. The cells were imaged in phase contrast with a 20x objective in a Nikon Eclipse TS100 light microscope equipped with Lumenera Infinity1 camera (Nikon Instruments Inc., Melville, NY). The beads-to-cell ratio was determined by direct counting on microscopic pictures using the MetaMorph image analysis program (Molecular Devices, Sunnyvale, CA.). The loading was confirmed by examining the light scattering by beads-loaded cells via flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). To visualize F-actin, cells were fixed with 4% paraformaldehyde, permabilized with Triton X-100, stained with AlexaFluor 488-phalloidin (all from Life Technologies, Waltham, MA), and observed with the Olympus FV 1000 Spectral confocal system (Olympus America Inc., Melville, NY) operated with the Olympus FV10-ASW software.

Magnetic pulldown in fibrous scaffolds. Fibrous scaffolds were prepared from poly-capro-lactone (PCL) by electrospinning, as described (Nam et al. 2007). They were of ~100 μm in thickness and fiber diameters were in 1-20 μm range (with a median of 10 μm). The scaffolds were cut and glued at the edges on glass microscopy coverslips, then placed in 24-well tissue culture plates for cell pull-down and image analysis.

The scaffolds were sterilized with 70% ethanol, washed twice with sterile phosphate buffered saline (PBS), washed once more with EGM-2 complete cell culture medium and finally overlayed with 1 mL of cell culture medium. 250,000 cells, either unlabeled, labeled with VEGFR-2 conjugated beads, or labeled with unconjugated beads, were suspended in 1 mL medium, added on top of the existing fluid, and incubated at room temperature for either 5 or 20 minutes, on a magnet with a peak strength of 0.4 T (LifeSep, model 96F, Dexter

Magnetic Technologies, Elk Grove Village, IL). After incubation, and while still on the magnet, the supernatant was removed, and the scaffolds were taken out and cut in half with a sharp razor blade. One half was fixed in 4% paraformaldehyde, stained with Alexa-488-phalloidin for actin demonstration as described (Jones et al. 2015), re-mounted on glass coverslips in Fluoromount™ (Sigma-Aldrich, St. Louis, MO) and imaged with the Olympus FV1000 Spectral confocal system. The other half of each scaffold was frozen *in situ* in the plate at -80°C for DNA extraction and quantification.

Cell number estimation. Actual cell numbers were determined in confocal images of the scaffolds using a custom image analysis software capable of accurate segmentation of the cell nuclei (Pecot et al. 2012). For validation, DNA was extracted from scaffold-attached cells by heating at 56°C for 30 min in 200 µL of a mixture of 100 µL PBS, 100 µL AL Buffer, and 10 µL proteinase K (DNeasy Blood & Tissue Kit, Qiagen, Valencia, CA). For the standard curve preparation, a 1:2 serial dilution of 125,000 cells was prepared and cells were lysed in the same manner. DNA was measured by a Pico-Green based assay (Quant-iT™, Invitrogen, Grand Island, NY).

Statistics. The distribution and resulting mean of beads/cell for seeded cells was calculated, and the results of the pull-down experiments were analyzed by ANOVA, Tukey HSD, and Kruskal-Wallis Test, using the JMP statistical program (version 12.2.0, SAS Institute, Inc. Cary, NC.).

RESULTS

Binding and internalization of microbeads by EC in suspension. To this end, we conjugated anti-biotin-covered MACSiBeads with a biotinylated anti-VEGFR2 antibody and incubated them with EC in suspension. During a 30-min incubation, the beads became strongly associated with the cells (**Fig. 1a**). However, due to the multiple binding sites on both cells and beads, this attachment led to massive clumping of the cells (**Fig. 1a**). These clumps could be partially re-dispersed by vigorous pipetting, but this is an inefficient, poorly reproducible, and time-consuming process, while producing bead detachment and likely cell damage (**Fig. 1b**). When examined in confocal microscopy, we could see many beads within the cells (**Fig. 1c**), in addition to those on the surface. Notably, beads were found in compartments surrounded by polymerized actin (**Fig. 1d**), which is a hallmark of phagocytosis (Marie-Anais et al. 2016). Therefore, although HUVEC in suspension bound and

actively internalized magnetic beads, the fact that they were also clustered limited their use in some applications, which prompted us to explore an alternative labeling approach.

Phagocytosis of microbeads by attached EC in monolayer culture. We reasoned that if the beads were already internalized, the labeled cells could be dispersed *after* trypsinization and pipetting, without concern for cell aggregation and/or additional bead loss. To this end, we incubated HUVEC cultured on tissue culture treated plastic with both VEGFR2 antibody-conjugated and -non-conjugated (control) beads, for 24 h. Considering that many physiological properties of confluent, quiescent cells are different from those of sub-confluent cultures, which are actively dividing and/or migrating, we also compared the beads' interaction with both confluent and sub-confluent HUVEC. In confluent cells, the beads initially settled under gravity mostly at the intercellular junctions (**Fig. 2a**, Day 0). After 24 h, the beads apparently redistributed across the entire cell and often displayed a peri-nuclear localization, suggesting their restriction in an endocytic compartment (**Fig. 2c, d**; compare with **2a** and **2b**). In confluent cultures, neither the beads' density nor their spatial distribution changed substantially after thorough washing, indicating that the beads strongly interacted with the cells or were internalized (**Fig. 2c**). However, fewer beads remained associated with the cells after washing in the sub-confluent condition (**Fig. 2d**). In both cases, the presence of the antibody on beads led to slightly more beads interacting with the cells. In confluent cells, we found an average of 4.2 unconjugated beads per cell and 4.4 beads per cell when they were conjugated with the VEGFR2 antibody. In the sub-confluent condition, cells incubated with non-conjugated beads contained an average of 2.9 beads, versus an average of 5.2 beads for sub-confluent cells incubated with beads conjugated with VEGFR2 (**Fig. 2c**, **d** and below).

To further verify whether the beads were permanently attached to, or internalized within cells, we performed cell trypsinization. This was expected to release the beads by digestion of the binding sites, if they were only attached to the cell surface. In practice, the beads remained associated with cells after trypsinization and even upon re-seeding in culture for further expansion. However, their numbers were visually reduced on a per-cell basis after passage (**Fig. 2e, f**).

We further analyzed by flow cytometry the cells after their incubation with the beads. Since the beads associated with cells were expected to change their light-dispersing properties, this procedure conveys further

information about the stability of cell-beads association, a method previously used to assess endothelial phagocytosis (Fens et al. 2010; Gao et al. 2013). In the 'side-scattering' analysis mode (SSC), the signal is proportional with light dispersion on objects, indicative of their granularity (in our case, due to beads association with the cells)(Ozdogu et al. 2007). Indeed, the comparison of side-scattering histograms showed that this parameter was increased upon incubation with the beads (from a median of 92 for HUVEC to >200 for all HUVEC with beads ± antibody) (**Fig. 3a**, compare control HUVEC with all other histograms, and **Fig. 3b** for direct counting). Consistent with direct phase-contrast microscopic examination, for both sub-confluent and confluent HUVEC, light dispersion was larger when the beads were bound to cells via VEGFR2 (**Fig. 3a**, compare green dotted with blue histograms for subconfluent cells, and purple dotted with red histograms, for confluent cells).

For practical applications, it is beneficial to perform a pre-separation of labeled cells from unlabeled ones. This was accomplished by enriching the suspension in beads-containing cells by a lateral magnetic holder, which attracted the magnetically-responsive particles to the walls of the container, while the non-magnetic ones, or those experiencing a smaller force, settled to the bottom. As a consequence, in flow cytometry, the magnetically collected (bead-enriched) cells displayed an increased side scatter signal when compared to the cells that settled in the tube without being retained in the magnet (an increase of the median from ~140, comparable to HUVEC control, to ~290 for magnetically collected cells) (**Fig. 4a**). After selection, the difference in average labeling efficiency (beads/cell distribution, determined by direct counting) between cells containing antibody-conjugated and non-conjugated (control) beads tended to vanish (**Fig. 4b**). Removal of non-labeled cells by lateral magnetic separation effectively raised the number of beads/cell to approx. 5.2 (although this difference was not significant as compared to the pre-separation beads/cell values in confluent conditions, $p = 0.0626$).

Magnetic collection of beads-loaded cells into a porous scaffold. Next, we applied this labeling method for seeding EC into fibrillar PCL scaffolds, by direct pull-down. The scaffolds consisted of meshes of random fibers with a median diameter of 10 μm, as previously determined from three-dimensional confocal images (Park et al. 2013). Suspensions of cells labeled with beads conjugated or unconjugated with the VEGFR2

antibody, and pre-sorted on a lateral magnet (to select labeled cells only), as well as suspensions of cells not labeled with beads as controls, were added on top of scaffolds attached to 24-well tissue culture plates.

We found that most of the cells were magnetically collected from suspension within the first 5 min, as the number of cells did not change with further incubation to 20 min (32.1+/-11.1 vs. 33.4+/-10.6 cells per microscopic field ($p > 0.05$), respectively, as determined with an custom-made imaging analysis software (Singh et al. 2011). Most of the cells remained attached to the fibers or trapped within the scaffold (**Fig. 5a**), either in the virtual pores randomly created between fiber crossings, or as small clusters forming bridges between the fibers (**Fig. 5b**). Microscopically, the only additional change observed with longer incubation time was the progression of cell attachment to the fibers, sometimes manifested as cell elongation (compare **Fig. 5c** with **5a**). HUVEC in the process of adherence also displayed a robust cell structure even in the presence of internalized beads, as indicated by F-actin distribution (**Fig. 5d**).

DNA extraction from scaffolds indicated that as long as the majority of non-labeled cells were removed during the magnetic pre-selection, the use of antibody to conjugate the beads to cells did not bring an additional pull-down benefit (**Fig. 6**). It also suggested that the labeled cells have been magnetically collected from suspension during the first 5 min, because the additional time on the magnet did not increase the detected DNA in the scaffold ($p = 0.99$ for 5 vs. 20 min for cells incubated with beads conjugated with antibodies, and $p = 0.97$ when the beads were not conjugated with antibodies). However, as expected, the non-labeled cells continued to settle gravitationally after the 5 min collection period, with a significant difference in cells collected between 5 and 20 minutes ($p < 0.05$), although the cell numbers were significantly smaller at both time points (**Fig. 6**).

Discussion

In this study we present a versatile method for loading EC with relatively large magnetic microbeads, based on angiophagy, namely the ability of EC to internalize micron-sized (~3.5 μm) beads attached to culture dishes or in suspension, a function that has been less exploited for bio-technological applications so far.

EC are part of the reticulo-endothelial system (Wake et al. 2001), which also comprises the tissue macrophages and liver Kupffer cells (Chrastina et al. 2011), and are known to engulf other particles such as

apoptotic cells (Fens et al. 2010) and bacteria (Rengarajan et al. 2016). This phagocytic-like activity also helps the clearing, by EC, of occluded microvascular lumens (Grutzendler 2013). In one of our previous works angiophagy was indicated by the presence of characteristic surface-located 'phagocytic cups' and of actin-rich phagosomes around microbeads (Jones et al. 2015).

Here we found that although the number of bound and internalized beads was moderately enhanced by coating them with the ligand of a cell receptor (VEGFR2) which is distributed on their apical surface (Stefanini et al. 2009), EC also efficiently bound and internalized control beads that were not antibody coated. In some publications this process was assumed to be ligand-dependent (e.g., lactadherin)(Fens et al. 2008), by a mechanism called 'opsonization' (Hart et al. 2004). However, in all previous situations the non-opsonized particles used as controls were also actively taken up *in vitro* by EC (usually HUVEC), albeit at about 1/3 of the opsonized particles rate (Gao et al. 2013). In a recent reconsideration of the subject, angiophagy was found to be largely, albeit not totally, ligand-independent and specifically controlled by the actin-nucleating formins FHOD1 and FMNL3 (Rengarajan et al. 2016).

It is still possible that bead engagement through their binding to surface molecules, such as VEGFR2, could provide a proliferative and/or survival advantage versus the non-conjugated beads, or could be purposefully used to stimulate them (Chretien et al. 2010; Collins et al. 2012; Mahajan et al. 2017).Regardless, these magnetic beads, as currently used, did not seem to adversely affect cell survival or proliferation, unlike the slight toxicity of magnetic nanoparticles (Pislaru et al. 2006a). Moreover, we found that after reseeding the labeled cells, the number of beads per cell decreased over time, as the cells replicated. This spontaneous 'dilution' of the beads (and possibly their active unloading) during cell division, is useful for the anticipated medically-oriented tissue engineering applications of the method.

The ability of EC to efficiently internalize beads without antibodies has potential benefits with respect to preparation time, increased simplicity, and reduced cost of cell labeling with beads. In our study, a key improvement to previous labeling methods is the dispersion of beads-labeled cells *after* the beads were internalized by adherent EC. This approach prevented bead-induced cell aggregation, a serious drawback of previous suspension-based cell labeling methods. To magnetically select EC from heterogeneous mixtures, EC

were previously surface-labeled with lectin (Jackson et al. 1990;Ishii et al. 2011) or albumin (Consigny et al. 1999) -coated microbeads, or with beads conjugated with anti-CD31/PECAM-1 antibodies (Springhorn et al. 1995). In these instances, the cell-bead interaction was performed directly in suspension. This procedure commonly leads to cell clumping, although this was acceptable in those instances, because the cells were then simply re-plated for further cultivation. But even in this case cell aggregation made difficult the determination of beads/cell ratio, as well as isolation of single cells, if needed. Substrate-attached EC were also labeled for cell tagging by spontaneous internalization of much smaller magnetic nano-particles, followed by cell dispersion (Pislaru et al. 2006b). To the best of our knowledge, direct uptake of micron-sized magnetic beads by attached EC has not yet been thoroughly studied, except incipiently in another publication (Mahajan et al. 2017).

Magnetic pre-selection of labeled cells reduced the distinction between labeling efficacy in the presence or absence on the beads of antibodies targeting cell surface ligands. This observation is compatible with the uptake of albumin-coated beads by EC (Consigny et al. 1999), and furthers the evidence of a lower cost cell loading alternative.

The beads-loaded cells were rapidly (< 5min) collected and seeded within electrospun scaffolds, by using a magnetic field of moderate intensity (0.4 T) produced by a flat commercial magnet. In terms of the efficiency of pulldown technique itself, the labeled cells were collected on the magnet faster into the scaffold than the control cells settling by just gravity. Since there was no statistical difference between the efficiency at 5 versus 20 min, we may assume that the magnetic cell collection was completed in less than 5 min.

In another study we also showed that HUVEC attached to such scaffolds were able to grow and differentiate during two weeks in culture (Jones et al. 2015). Longer-term (for more than a week) incubation of EC with this scaffold led to their intimate adhesion to fibers, and to their proliferation and differentiation (Jones et al. 2015). Thus, the compatibility of this particular scaffold with the EC recommends it as a valuable carrier for EC at sites of deployment.

Besides cell labeling with magnetic (or possibly fluorescent) beads, our findings describing the avid incorporation of microbeads by attached EC have various practical implications, for example in basic endothelial

biology (such as the clearing and transport of particles by EC), in bioengineering (e.g., for intracellular molecular delivery), and in tissue engineering (for controlled cell placement, as shown here).

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Figure legends

Fig. 1 Attachment and internalization of magnetic beads by HUVEC in suspension. a. Aggregates of HUVEC induced by incubation with anti-VEGFR2 conjugated, multivalent Anti-biotin MACSiBead (arrows). **b** Dispersion of cells after incubation and separation from non-labeled cells using the magnetic force; note that in this case all cells contain at least one bead, but some are heavily loaded (arrowheads, and insert). **c**. Serial optical sectioning of one cell demonstrates full internalization of the beads; and their enclosure within F-actin bordered phagosomes (Alexa 488-phalloidin, green). **d**. Highlighting of F-actin around internalized beads in the same images as in **c**. In **c** and **d**, the left images are phase contrast and rest are z-stack serial fluorescence microscopy sectioning through the same cell (HUVEC) incubated with anti-VEGFR2 beads, followed by fixation, permeabilization, and staining with Alexa 488-Phalloidin (green); beads are stained with Alexa 543-labeled anti-mouse antibody (red); nuclei were stained with DAPI (blue). Magnification bars: **a, b**: 50 μm; **c, d**: 10 μm

Fig. 2 Binding and uptake of magnetic beads by substrate-attached HUVEC. a-b. Beads initially settling over a confluent or sub-confluent HUVEC culture, respectively; in **A**, note their localization at cell borders. **c-d**. Association of beads after 24 h with confluent or subconfluent cells, respectively (upper row) and after thorough washing (lower row); the beads were either unconjugated (left columns, Beads only) or conjugated with the anti-VEGFR2 antibody (right columns, Beads + aVEGFR2). **e-f**. Beads-loaded confluent HUVEC after trypsinization and re-plating for an additional 24 hrs: **e**, non-conjugated beads; **f**: VEGFR2-conjugated beads. Note the intracellular localization of beads, and the dilution effect. Phase contrast microscopy. Magnification bars: 50 μm

Fig. 3 Assessment of bead loading within substrate-attached HUVEC. a. Flow cytometric determination of light side scattering (SSC-H), as an indirect measure of cell granularity (i.e., loading with particles). In this case, note the highest efficiency of uptake (i.e., side scatter) by confluent cells incubated with anti-VEGFR2 conjugated beads. Stability of cell-associated particles during trypsinization and flow cytometry analysis is an argument that they are strongly attached and/or internalized. **b**. Distribution of cell-associated beads obtained by direct counting.

In this experiment, labeled and non-labeled cells were not separated by magnetic pre-selection. Color code same as in **a**

Fig. 4 Effect of magnetic pre-selection on loading of HUVEC with beads. a. a. Light side-scattering measured by flow cytometry shows a higher granularity of the cells, meaning more associated beads, in the cells retained after pre-selection (blue lines; arrow) versus those remaining in suspension (red lines; arrowhead). Two replicate experiments are shown in each case. Control: bead-free cells (grey). **b, c**. Comparison of magnetic beads distribution in post-selection cells as dependent on the presence (**b**) or absence (**c**) of the anti-VEGFR2 antibody on the beads. The counting of beads/cells values was performed manually on microscopic images

Fig. 5 Detection of magnetically-collected cells within scaffolds. a. Representative image of the 5 min pull-down experiment; the collected cells were visualized by staining for F-actin (green), and the nuclei with DAPI (blue). **b**. At this time point, the beads-labeled cells were already either attached to or became trapped in between fibers, forming 'bridges' of a few cells (also in insert). **c**. Different field, illustrating the distribution of single cells (arrowhead) and clusters (arrow) on, and among fibers. **d**. After 20 min, the bead-labeled cells were found better attached and spreading along the fibers, by re-organizing their actin cytoskeleton, as an indication of their functionality (arrows, insert). Bars: **a, c, d**: 50 μm; **b**: 20 μm

Fig. 6 Assessment of magnetic cell collection efficiency. We compared by DNA analysis the two labeling conditions (with and without the anti-VEGFR2 antibody, followed by magnetic pre-selection), and the two time points (5 and 20 min), for cell collection in the scaffold. No significant differences were seen between the cells labeled with antibody-conjugated beads and those labeled with unconjugated beads at either time point, indicating that the pull-down process was completed in 5 min. However, at each time point there was a difference between the unlabeled, gravitationally settling control cells and the experimental groups (*$p<0.01$). *Inset*, the DNA standard curve