Epidermal Growth Factor – based adhesion substrates elicit myoblast scattering, proliferation, differentiation and promote satellite cell myogenic activation

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ARTICLE INFO

Keywords:
Elastin-like polypeptides
Epidermal Growth Factor
Cell adhesion
Satellite cells
Skeletal muscle regeneration
Biomimetic materials

ABSTRACT

The biochemical properties of muscle extracellular matrix are essential for stem cell adhesion, motility, proliferation and myogenic development. Recombinant elastin-like polypeptides are synthetic polypeptides that, besides maintaining some properties of the native protein, can be tailored by fusing bioactive sequences to their C-terminal. Our laboratory synthesized several Human Elastin-Like Polypeptides (HELP) derived from the sequence of human tropoelastin. Here, we developed a novel HELP family member by fusing the elastin-like backbone to the sequence of human Epidermal Growth Factor. We employed this synthetic protein, named HEGF, either alone or in combination with other proteins of the HELP family carrying RGD-integrin binding sites, as adhesion substrate for C2C12 myoblasts and satellite cells primary cultures. Adhesion of myoblasts to HEGF-based substrates induced scattering, decreased adhesion and cytoskeleton assembly; the concomitant presence of the RGD motifs potentiated all these effects. Recombinant substrates induced myoblasts proliferation, differentiation and the development of multinucleated myotubes, thus favoring myoblasts expansion and preserving their myogenic potential. The effects induced by adhesion substrates were inhibited by AG82 (Tyrphostin 25) and herbimycin A, indicating their dependence on the activation of both the EGF receptor and the tyrosine kinase c-src. Finally, HEGF increased the number of muscle stem cells (satellite cells) derived from isolated muscle fibers in culture, thus highlighting its potential as a novel substrate for skeletal muscle regeneration strategies.

1. Introduction

The homeostasis of skeletal muscle necessarily requires the sustained cross-talk between the extracellular matrix (ECM) and the cell cytoskeleton, which is essential to stabilize the muscle cell membrane under force transmission [1]. The evidence that mutations altering the interplay between cells and ECM invariably lead to functional impairment and muscle waste, such in several types of muscular dystrophies, dramatically highlights the crucial role of these interactions [1]. Besides conveying structural support, ECM is now recognized as a fundamental network enriched in extracellular signals highly active in the modulation skeletal muscle cells physiology [2]. ECM nests adhesion sites for a large number of growth factors, thus increasing their local concentration; this creates an environment endowed with survival, trophic and stimulatory signals capable of driving muscle cell behavior. Linking growth factors to ECM may ensures persistent activation of signaling pathways in target cells and tissues. Moreover, the presentation of growth factors in an immobilized form is likely to have important physiological consequences since soluble, membrane-anchored and matrix-linked growth factors may produce different effects in the *in vivo* environment [3–6]. Several lines of evidence point to immobilized EGF as a possible modulator of muscle development and regeneration. First of all, many ECM proteins crucially involved in myogenesis, like laminin, contain several EGF-like domains exerting growth factor activity [7]. Secondly, the presence of EGF-like repeats in agrin, an extracellular protein directing neuromuscular junction formation [8], is required to regulate the clustering of acetylcholine receptors [9] and is thought to modulate neuronal recognition [10]. Finally, membrane-

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Accepted 16 October 2018

anchored Heparin-Binding EGF-like Growth Factor (proHB-EGF), an alternative ligand for EGF receptor (EGFR) [3], has been indicated as a survival signal in differentiating C2C12 cells [11]. The persistent activation of EGFR throughout differentiation, together with the upregulation of membrane-bound HB-EGF, have been show to protect differentiating myoblasts from pro-apoptotic cues [11].

The possibility that immobilized EGF could influence the adhesion, survival, and differentiation of skeletal muscle cells has been addressed in the present study. Recombinant proteins mimicking human tropoelastin (Human Elastin-Like Polypeptides; HELPs) have been synthesized in our laboratory [12,13]. When employed as adhesion substrates for muscle cells they stimulated *in vitro* myogenesis [14–16]. To provide myoblastic cells with both survival and adhesion signals, we synthesized HELP-EGF (HEGF) and different RGD containing HELP fusion proteins, which were employed as adhesion substrates for C2C12 myoblasts and satellite cells primary cultures. The effects of their different sequences on cell morphology, adhesion, cytoskeleton, proliferation and differentiation have been compared. The results obtained indicate that adsorbed HEGF and RGD-containing proteins increased motility, proliferation and differentiation of C2C12 myoblasts and stimulated the activation and myogenic development of satellite cells from isolated muscle fibers.

2. Materials and methods

2.1. Antibodies and fluorochromes

Anti-vinculin antibody (mouse monoclonal anti-vinculin, V9131, Sigma-Aldrich, St. Louis, MO) was employed at 1:200 dilution. Phalloidin Atto-594 (51,927, Sigma-Aldrich) was employed at 4.5 pmol/coverslip. DAPI (4′,6-diamidino-2-phenylindole, D9542, Sigma-Aldrich) was employed at 200 ng/ml. Antibody against Myosin Heavy Chain (rabbit polyclonal antibody, H-300, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:30 dilution. Antibody against Ki-67/MKI67 (rabbit polyclonal antibody NB500/-170, Novus Biologicals, Littleton, CO) was employed at 1:30 dilution. Antibody against myogenin (mouse monoclonal antibody, sc-52,903, Santa Cruz Biotechnology) at 1:20 dilution. Antibody against Pax7 (mouse monoclonal antibody MAB1675, R&D Systems, Minneapolis, MN) was employed at 1:20 dilution. Goat anti-mouse fluorescein isothiocyanate (FITC) antibody, (F0257, Sigma-Aldrich) at 1:50 dilution. Alexa Fluor 488-conjugated AffiniPure goat anti-mouse IgG, (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:100 dilution. Alexa Fluor 594-conjugated AffiniPure goat anti-mouse IgG, (Jackson ImmunoResearch Laboratories) at 1:100 dilution. Goat anti-rabbit fluorescein isothiocyanate (FITC) - conjugated affinity purified IgG (Jackson ImmunoResearch Laboratories) was employed at 1:200 dilution.

2.2. HELP derivatives design and synthesis

The synthesis of HELPs was carried out as previously described [13]. Synthesis of HELPc has been already detailed as well [15]. The scrambled derivatives of HELPc, Hscra and HRGD, were generated by Sequence Manipulation Suite [17]. For the synthesis of HEGF, the synthetic gene of the HELP polypeptide was fused with the 53aa coding sequence of the human Epidermal Growth Factor (GenBank AAS83395.1), exploiting the unique DraIII site in the expression vector that allows the in-frame insertion at the C-terminus of the polypeptide [13]. All the final constructs were sequence-verified.

The recombinant products were expressed in the C3037 *E. coli* strain (New England Biolabs, Ipswich, MA). Expression and purification were carried out as already detailed [18]. Optimal HEGF purification required a modification of the protocol. Briefly, the pellet obtained from 1.2 l of IPTG-induced bacterial culture was re-suspended in 400 ml of extraction buffer (50 mM Tris/HCl pH = 8, 250 mM NaCl, 0,1 mM EDTA, 0,1% Triton X-100, 1 mM PMSF) and disrupted using a high pressure homogenizer (Panda NS1001L, GEA Niro Soavi, Italy). The recovered suspension was cooled on ice, added with 20 mM 2-mercaptoethanol, and centrifuged at 10000 rpm for 30 min at 8 °C (Beckman–Coulter, J-26 XP). Supernatant was properly diluted by adding fresh extraction buffer and precipitated with half a volume of 5 M NaCl, at 42 °C for 10 min. Aggregated polypeptide particles were pelleted by centrifugation at 10000 rpm, 39 °C for 30 min. The pellet was re-dissolved in a solution of 1 mM sodium taurocholate, 0.05% (v/v) Tween-20, 0.1% (v/v) 2-mercaptoethanol; non-soluble material was discarded by cold centrifugation. The supernatant was re-precipitated by addition of half a volume of 5 M NaCl. After this last temperature-dependent transition cycle, the purified polypeptide was re-suspended in water and frozen. All recombinant products were analyzed by SDS-PAGE and the purified products were lyophilized for long term storage.

2.3. HELP polypeptides coating

Borosilicate glass coverslips (VWR International, Milano, Italy) where cleaned by extensive (overnight) stirring in acetone followed by an extensive wash with absolute ethanol. Coverslips sterilization was obtained by dry heating at 120 °C. For coatings, the lyophilized polypeptides were re-dissolved in water and sterilized by filtration (0.22 μm). Protein concentration was verified by the Bradford method and adjusted to the desired level by dilution. For experiments with C2C12 cells, HELPs coatings were obtained by depositing 100 μl of 0.1 mg/ml coating solution per 1 cm^2 surface area that was incubated for at least 2 h at 37 °C. Just before cell seeding, the solution was removed by aspiration and coverslips washed with PBS. Similar procedure was applied to 96 wells microtiters employed for WST-1 assays.

For muscle fibers seeding, borosilicate glass coverslips were coated with Matrigel (Corning, Corning, NY) in the absence and in the presence of HELP or HEGF. Growth factor reduced Matrigel stock solution (1:50 in DMEM, 1 mg/ml final concentration) was employed. HELP/Matrigel and HEGF/Matrigel were prepared by mixing 1 vol of HELP (or HEGF, both at 0.1 mg/ml) to 4 vol of Matrigel stock solution. The control coating solution was obtained by mixing 4 parts of stock solution with 1 part of sterile water. Coating was performed by depositing 100 μl of the coating solution on coverslips maintained on an ice plate. After 10 min the excess solution was removed and the adhering protein layer was allowed to settle for 30 min at 37 °C. Coated coverslips were then extensively washed with PBS, to remove unadsorbed protein.

2.4. X-ray photoelectron spectroscopy analysis

To assess the proper coating of the coverslip supports X-ray photoelectron spectroscopy (XPS) was performed. Both polystyrene (Sarstedt AG&Co, Nümbrecht, Germany) and glass (VWR International, Milano, Italy) coverslips were coated as described in the previous paragraph. After incubation with the protein solution, the excess was removed by aspiration and coverslips quickly rinsed with sterile water. They were then incubated overnight in water (at 37 °C) to wash away any unadsorbed protein. For comparison, in some coverslips the protein solution was simply allowed to dry, omitting the washing steps. The analyses were performed using an ultra-high vacuum surface analytical tool equipped with SPECS Phoibos 150 hemispherical analyzer. The applied voltage of the Mg Ka X-ray source was set at 12.5 kV and the applied current at 15 mA. The background pressure in the analysis chamber was 2×10^{-8} Pa. For the wide scan, the pass energy was 40 eV, the energy step was 0.5 eV and the scan number was 1. For the narrow, high resolution scan, the pass energy was 20 eV and the energy step was 0.05 eV. Surface charging was corrected by reference the spectra to the C 1s signal of aliphatic C—C carbons located at 285.0 eV. The spectra were then analyzed using KolXPD software [http://www.kolibrik.net/science/kolxpd/]. For fitting, Voigt profile was used, after subtraction of a Shirley-type background.

2.5. C2C12 culture

The mouse myogenic C2C12 cells were maintained as exponentially growing myoblasts in a Growth Medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), GlutaMAX supplemented (Gibco, ThermoFisher, Waltham, MA) added with 20% heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were cultured on 10 cm polystyrene Petri dishes at 37 °C in a 5% CO_2 incubator. Cells were serially passed at about 75% confluence every 2–3 days. For adhesion and scattering evaluation, cells were plated at a density of 10^4 cells/cm^2, and allow to settle for 24 h. When necessary, trypsinized C2C12 myoblasts were seeded in the presence of AG82 (α-Cyano-(3,4,5-trihydroxy)cinnamonitrile, 10 µM), Herbimycin A (0.5 µM) (both from Calbiochem, La Jolla, CA), human Epidermal Growth Factor (2 nM, Sigma-Aldrich) or soluble HEGF (2 nM).

To obtain cell differentiation and myotube fusion into myotubes, cells were plated at a density of 5×10^3 cells/cm^2. 48 h after plating, cells were shifted to Differentiation Medium, consisting of DMEM, GlutaMAX supplemented, added with 2% heat-inactivated horse serum, 1 µM insulin, 100 IU/ml penicillin and 100 µg/ml streptomycin. Myotubes were allowed to develop for 4–6 days.

2.6. Immunofluorescence

The general protocol for immunofluorescence experiments is described. Coverslips were washed three times with Phosphate Buffered Saline (PBS) and then fixed with paraformaldehyde. Cell fixation was performed with 4% paraformaldehyde for 20 min at 4 °C, and samples were then washed three times with PBS (10 min each). After fixing, samples were blocked with a solution containing 1–5% normal goat serum, 0.1% Triton-X 100 in PBS for 10 min. Samples were incubated overnight at 4 °C with a primary antibody at the appropriate dilution. After three washes of 10 min with PBS added with 0.1% Triton-X 100, coverslips were incubated with the secondary antibody (or other fluorochromes) for 2 h at 4 °C. Coverslips, washed three times for 10 min with PBS/ 0.1% Triton-X100, were then mounted onto slides and visualized under a Leica DMLS fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Images were acquired with a Leica DC300F camera, coupled to a Leica LM50 acquisition software. Image sizing, cropping and overlays were obtained with Adobe Photoshop CC (Adobe Systems Incorporated, San Jose, CA). Image analysis, measurements of cell areas, cell and nuclei counting were performed employing ImageJ [19]. At least 10 microscope fields in three different samples for each experimental condition were analyzed.

2.7. WST-1 assay

C2C12 were cultured in 96 multiwell plates coated with the different HELP polypeptides. Metabolic activity was evaluated with the WST-1 (2-(4-iodophenyl)-3-(4-nitophenyl)-5-(2,4-disulfophenyl))-2H-tetrazoilium monosodium salt, provided in a pre-mix electro-coupling solution (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Cell cultures were treated with 100 µl of culture medium added with 5 µl of the pre-mix solution for 2 h. Absorbance was read at 450 nm with a microplate reader (Synergy H1, BioTek, Winooski, VT). For estimating metabolic activity, cells were seeded in Growing Medium at a density of 5×10^3 cells/well, and the activity was evaluated at 5, 24 and 48 h. At 48 h samples where shifted to Differentiation Medium and metabolic activity evaluated at 72 h and 96 h. When required, soluble EGF and HEGF (both 2 nM) were present throughout the culture. To provide a dynamic description of the time-dependent increase in activity the WST-1 values were normalized to the optical density measured at 5 h, after blank subtraction.

2.8. Differentiation assays

C2C12 cells, were induced to differentiate for 48 h or 96 h. Cells differentiated for 48 h were processed for immunofluorescence against myogenin. Cells differentiated for 96 h were immunostained against Myosin Heavy Chain (MHC). A MHC-expressing cell containing 3 or more nuclei was considered as a myotube. The fusion index was calculated as the ratio of the nuclei number in MCH-positive myotubes *versus* the total number of nuclei in the field. The average number of nuclei per myotube was determined by dividing the number of nuclei in myotubes by the total number of myotubes.

2.9. Satellite cells culture

Experiments were carried out using *in vitro* preparations of muscle fibers associated with satellite cells and prepared from the *Flexor Digitorum Brevis* (FDB) muscles of 6 to 8-weeksold C57BL/6J male mice [20] with few modifications.

Mice were fed *ad libitum*. For muscle dissection, they were anesthetized and sacrificed by cervical dislocation. All animal procedures were conducted in accordance with the National Institutes of Health and with international and institutional standards for the care and use of animals in research. All experiments were performed in accordance with European Union (EU) guidelines (2010/63/UE) and Italian law (decree 26/14) and were approved by the Italian Ministry of Health.

FDB fibers with associated satellite cells were isolated from both hind feet of a single mouse for each preparation. Briefly, muscles were treated with Type I collagenase 0.3% (wt/v, Sigma-Aldrich) in Tyrode solution supplemented with penicillin 100 IU/ml, streptomycin (100 µg/ml, Euroclone, Pero, Italy) and Fetal Bovine Serum 10% (Gibco, Burlington, ON, Canada). Enzymatic digestion was carried out for 1 h at 37 °C. Connective tissue was removed washing the samples twice in Tyrode solution before proceeding to mechanical dissociation. Single muscle fibers were obtained by gentle dispersion of the samples using flame-polished Pasteur pipettes with decreasing tip diameters.

Myofibres released from FDB were allowed to settle 2 h on Matrigel-based glass coverslips coatings (see paragraph 2.3), accommodated in 35-mm plastic culture dishes. The differentiation medium for satellite cells was then added to cover the entire coverslip. Differentiation medium consisted of Dulbecco's modified Eagle's Medium (DMEM high glucose, Sigma-Aldrich) enriched with 5% Horse Serum HS (Sigma-Aldrich), L-Glutamine 2 mM (Euroclone), penicillin (100 IU/ml) and streptomycin (100 µg/ml, Euroclone). Typically, a preparation from one mice resulted in 15 to 18 coverslips with 45–50 myofibres per each of them. All dishes were maintained in incubator at 37 °C. Medium was replaced every 48 h.

2.10. Statistical analysis

Data were analyzed with OriginPro software (7.5, OriginLab Corp. Northampton, MA). Statistical significance was determined using one-way ANOVA analysis after Levene's test for homogeneity of variances, and followed by the Scheffè test for multiple comparison test. Unless otherwise stated, results were expressed as mean ± standard deviation (SD). Difference with $p < 0.05$ was considered as statistically significant.

3. Results

3.1. HELPs primary structures and coatings

The schematic structures and the aminoacid sequences of HELP polypeptides employed in this study are reported in Fig. 1a. The HELP backbone, derived from the human tropoelastin sequence, is composed by eight alternate repeats of two modules: an Ala-rich, α-helical domain, nesting two Lys residues, and a hydrophobic domain, composed

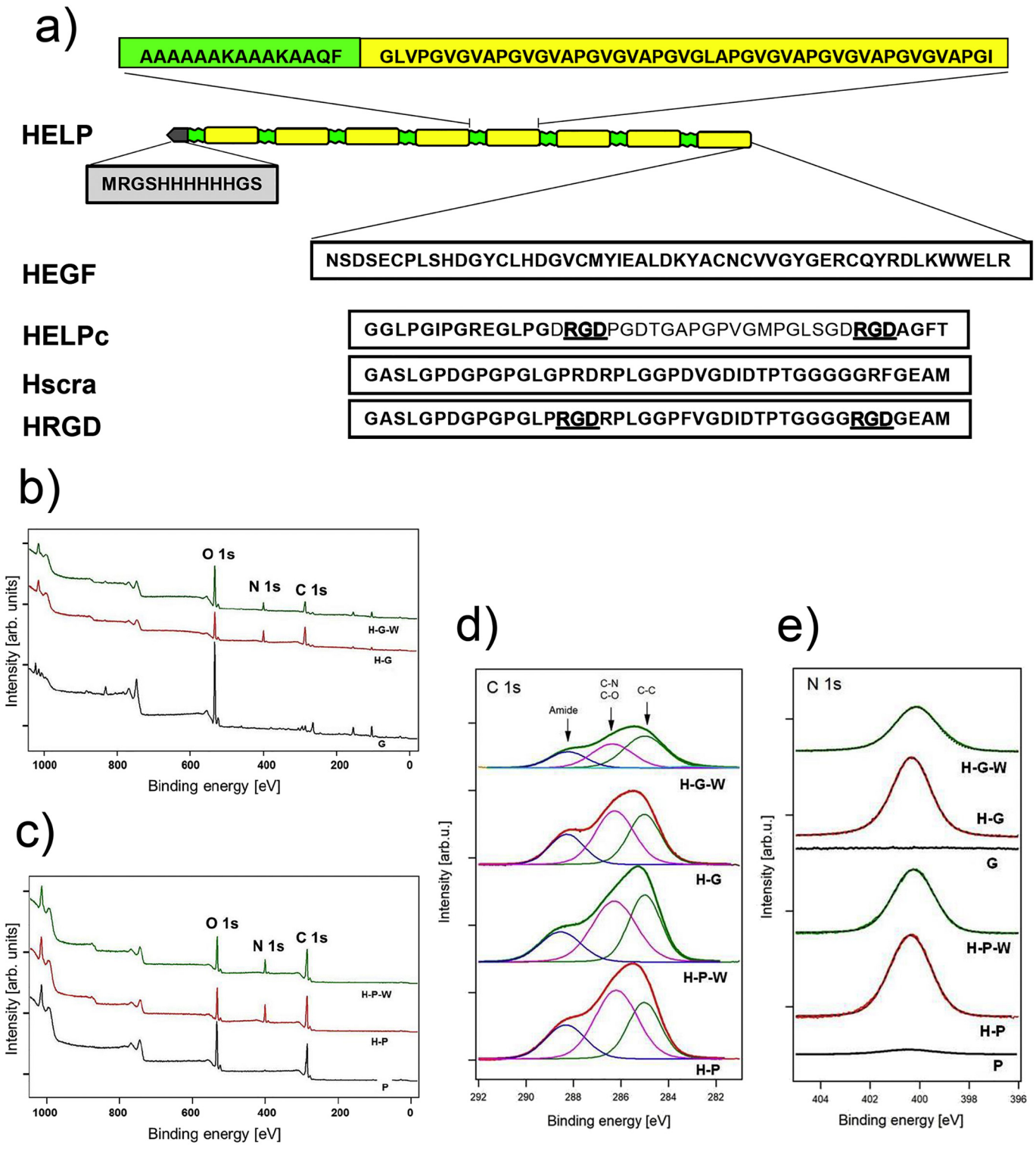

Fig. 1. HELP polypeptides primary structures and adsorption. a) Schematic representation of the HELP backbone. The aminoacid sequence of the Ala-rich (green) and hydrophobic (yellow) domains that compose the repetitive unit are reported. A His-tag sequence is located at the N-terminal portion (dark grey) of each polypeptide. HEGF was obtained by fusing the sequence of human Epidermal Growth Factor at the C-terminus of HELP. HELPc was obtained by fusing a 43aa sequence from the α2 chain of the type IV collagen at the C-terminus of HELP. In Hscra the whole sequence of type IV collagen was scrambled, while in HRGD the position of the original RGD motifs was maintained. The two RGD motifs of HELPc and HRGD are evidenced (bold). b) XPS survey spectra on borosilicate glass. The spectra of glass substrate (G, black) is compared to that HELP coatings that were immediately dried after protein adsorption (H-G, red) or subjected to extensive wash out to remove the unadsorbed protein (H-G-W, green); c) XPS survey spectra on tissue culture polystyrene coverslips. The spectra of the polystyrene (P, black) is compared to that of unwashed (H-P, red) and washed (H-P-W, green) HELP coatings. d) the evolution of C 1s XPS peak of washed (H-G-W, green) and unwashed (H-G, red) HELP coatings on borosilicate glass substrate was compared to that of washed (H-P-W, green) and unwashed (H-P, red) samples on polystyrene substrate; e) the evolution of N 1s peak of washed (H-G-W, green) and unwashed (H-G, red) HELP coatings on borosilicate glass (G, black) was compared to that washed (H-P-W, green) and unwashed (H-P, red) samples on polystyrene substrate (P, black).

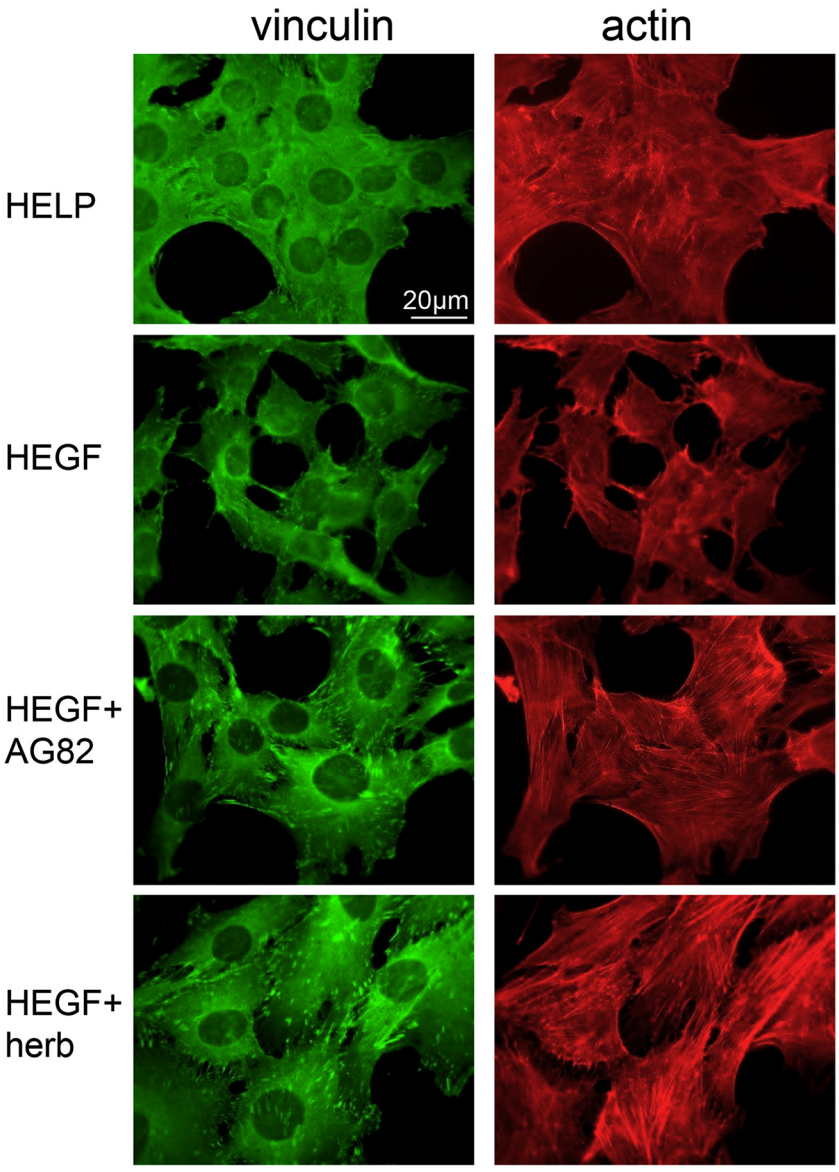

Fig. 2. Effect of adhesion substrates on myoblasts focal adhesion and actin cytoskeleton. C2C12 cells, seeded at a density of 10^4 cells/cm^2 were allowed to adhere for 24 h to glass coverslips coated with HELP and HEGF. Cells seeded on HEGF were cultured in the absence or in the presence of AG82 (10 µM) or Herbimycin A (0.5 µM). Immunofluorescence labeled vinculin (green), F-actin was labeled with Atto 594 phalloidin (red). The results are representative of four independent experiments done in triplicate (n ≥ 480 cells/condition).

by repetitions of hexapeptidic VAPGVG motif [12]. The elastin-like backbone was preceded by a His-tag sequence at its N-terminus. The other polypeptides were synthesized by fusing the sequence of interest at the C-terminal region. HEGF was obtained by fusing the 53 aa sequence of human Epidermal Growth Factor. HELPc, that has been already described, was synthesized by fusing a sequence of 43 aa from the α2 chain of type IV collagen, harboring two RGD motifs [15,16]. A scrambled sequence of these 43 aa has been fused to HELP to obtain the Hscra polypeptide. Finally, the HRGD construct carried the same 43 aa scrambled sequence, but maintained the two original RGD motifs. Adsorption of HELP protein on borosilicate glass and polystyrene coverslips was assessed by X-ray photoelectron spectroscopy (XPS). Uncoated glass and polystyrene coverslips were analyzed and compared to the same supports coated with the HELP polypeptide (100 µl of 0,1 mg/ml solution). Protein coating was allowed to dry either before or after extensive water rinsing (see Methods). Fig. 1b shows the survey spectra of borosilicate glass coverslips. The spectra of HELP samples deposited on the glass substrate and analyzed without the washing steps (H-G, Fig. 1b) show a clear contribution of nitrogen, an indirect evidence of protein adsorption [21]. The nitrogen signal remained clearly detectable in water rinsed samples (H-G-W, Fig. 1b), although its intensity was decreased to some extent. No nitrogen signal was detected in uncoated glass coverslips (G). This indicated that HELP polypeptide remained firmly adsorbed to the glass support even after extensive rinsing. Similar results were obtained for HELP deposited on polystyrene (Fig. 1c), confirming the observations already reported [14].

The evolution of C 1s peak of washed and unwashed HELP is shown in Fig. 1d. The spectra were fitted by three components corresponding to aliphatic carbon at 285.0 eV (used for spectra calibration), the peak at 286.2 eV that was assigned to the C—N and C—O bonds and, finally, the peak at 288.3 eV, assigned to the contribution from carbon atoms of the polypeptide backbone N—C═O or N—C═O—H [14,22,23]. The same evolution for N 1 s spectrum is shown in Fig. 1e. The position of the single component at the binding energy of 400.3 eV corresponded to expected value for peptidic nitrogen atoms [23,24]. Both the C 1s and N 1s spectra confirm the binding of HELP coating on both borosilicate and

polystyrene substrates. In the case of polystyrene, the reduction of peak intensity after washing was less pronounced than that on borosilicate glass suggesting that HELP adsorption on polystyrene was slightly more efficient. We detected a considerable peak broadening in the glass sample which underwent washing (H-G-W, Fig. 1d and e). The broadening towards lower binding energy, observable on C 1s (Fig. 1d), N 1s (Fig. 1e) and Si 2p (not shown) at binding energies of 283, 398.6 and 102 eV, could be assigned to formation of Si—C, N—O—Si and Si—O—N weak interactions, [25]. Overall, these data indicate that the HELP backbone stably adsorb to different surfaces suitable for cell culture.

In order to compare the adsorption of different protein constructs to borosilicate glass with a standard assay (Supplementary Materials and Methods), coverslips were coated with 10-fold concentrated HELPs solutions (1 mg/ml) to allow the evaluation of the amount of protein that remained attached to coverslips after extensive washing. Results, reported in Supplemental Fig. 1a, show that the different protein constructs, independently on their C-terminal sequence, similarly adsorb to glass coverslips, suggesting a major contribution of the elastin-like backbone to stability of the adsorbed protein layer. The possibility that the adsorbed protein layer could be removed by cell culture media was next assessed. The results indicated that incubation with cell culture media increased the amount of protein adsorbed to coverslips (Supplemental Fig. 1b). The amount of protein recovered from HELPs-coated samples was significantly increased, suggesting that HELPs coatings favor the adsorption of other proteins. This could be relevant during cell culture, when ECM proteins are synthesized and secreted by the cells. Under these conditions, besides providing biochemical cues modulating cellular activities, HELP and its derived variants could supply an anchoring factor, favoring the stabilization of ECM proteins, ultimately leading to increased myogenesis. Indeed, cell attachment to HELP polypeptides show increased morphological and functional differentiation with respect to glass adhesion [15].

Taken together, these results indicated that the HELP C-terminal fusion of bioactive domains could represent a versatile engineering strategy at the cell-substrate interface.

3.2. Cell adhesion

The primary structure of HELP polypeptides deeply impacts on adhesion, morphology and spreading properties of C2C12 cells [15]. Accordingly, vinculin- expressing focal adhesions and actin cytoskeleton properties, evaluated 24 h after cell seeding on HELP- and HEGF-coated glass coverslips, revealed interesting differences (Fig. 2). Cells seeded on HELP displayed a high degree of intercellular contacts, forming regions in which cells strictly adhere to each other. In these monolayers, vinculin localization appeared mainly diffused, with only few brighter spots localized at the plasma membrane. The actin cytoskeleton appeared only partially organized, with few actin filaments crossing the cells. Cortical actin layers were visible only in the periphery of monolayers, at the edges of cells interfacing the culture medium. Adhesion to HEGF induced substantial modifications in cell morphology, spreading and contacts, as well as an evident disassembly of the actin cytoskeleton, with disappearance of cortical actin. Cells acquired a stellate morphology and displayed massive reduction of intercellular contacts (scattering).

To investigate the role played by the EGF receptor (EGFR) signaling on HEGF-induced cell scattering, C2C12 myoblasts were seeded in the presence of AG82 (Tyrphostin A25), a widely employed EGFR inhibitor [26]. In parallel experiments, cells were incubated with herbimycin A, an inhibitor of the non-receptor tyrosine kinase c-src [27]. The effects of the two inhibitors were striking, albeit slightly different. Compared to untreated counterpart, cells incubated with AG82 (10 μM) spread more widely and show a substantial recovery of cell-to-cell contacts (Fig. 2 and Supplemental Fig. 2). Moreover, they display marked spots of vinculin positivity, indicating clustering of focal adhesions both at the monolayer periphery and at intercellular contacts. The inhibitor also induced the assembly of actin cytoskeleton, with layers of cortical actin and stress fibers crossing the cells soma. Herbimycin A induced even stronger effects: compared to untreated samples, cell spreading was markedly enhanced and intercellular contacts occurred through several, tiny filipodia branching off the plasma membrane. Focal adhesion clustered both at the cell boundary and throughout the cell soma and cytoskeleton appeared highly organized, with both thick layers of cortical actin and prominent stress fibers crossing the cell bodies. Taken together, these results indicated that on HEGF-coated substrates cell scattering occurred due to the stimulation of EGFR and to the involvement of the cytosolic tyrosine kinase c-src.

The effect of HEGF on cell adhesion, scattering and spreading depended on substrate concentration. Indeed, dilution of the HEGF coating solution by fivefold resulted in cell scattering inhibition (Supplemental Fig. 3). Since the inhibitory effect could be attributed to the decreased concentrations of the growth factor or to the reduced amount of the total protein, an alternative experimental strategy was pursued to clarify this issue. In a next series of experiments we coated coverslips with two-component HELPs solutions, obtained by mixing HEGF to the other HELP proteins at fixed ratios (1:5). Consequently, the total protein concentrations of the coating solutions was kept constant, but the amount of EGF was reduced by fivefold. Besides enabling fair comparison of the substrate coating, this strategy also allowed us to assay the effects of adhesion signals on HEGF-induced responses. Indeed, integrin-mediated adhesion to ECM components regulates the cellular responses to growth factors, which, in most cases, is anchorage-dependent [28]. The signal transduction events activated by EGF are known to synergize with those triggered by integrins engagement and a bidirectional cross-talk between the two pathways characterizes several cell types [29–31]. Cell adhesion to HELP and to HEGF/HELP combined substrate is shown in Fig. 3. On the HEGF/HELP coating, the observed scattering effect of HEGF was totally lost and cell adhesion, spreading and cytoskeleton appeared indistinguishable from those observed with HELP alone. Cells adhesion to HELPc, which harbors the type IV collagen sequence at its C-terminus, induced extensive cell-to-cell contacts enriched with localized spots of vinculin positivity that were distributed both at the cell periphery and throughout the cell soma. The actin cytoskeleton was highly organized, with numerous stress fibers aligning mainly along the major cell axis and layers of cortical actin visible both at the cell-to-cell and cell-to-substrate boundaries. Intriguingly, in contrast to what observed for HEGF/HELP substrate, adhesion to the combined HEGF/HELPc substrate induced cell scattering similar to that induced by pure HEGF. However, compared to those seeded on pure HEGF, the scattered cells adhering to HEGF/HELPc substrate displayed focal adhesion and a higher degree of cytoskeleton assembly. To investigate the role played by the different segments of the collagen type IV sequence in modulating the effects of HEGF on cell adhesion, we employed the two different HELPc scrambled counterparts, Hscra and HRGD, described in Fig. 1a. Both Hscra and HRGD allowed cells to contact each other, forming monolayer regions and extensive cell-to-cell contacts (Fig. 3). However, HRGD considerably increased cell spreading, induced a higher number and size of focal adhesion and promoted the appearance of filipodia emerging from the plasma membrane. Moreover, HRGD was more effective in inducing the assembly of stress fibers crossing the cells along the main axis. Notably, although both HELPc and HRGD carry two RGD motifs in the same position, HRGD allowed more extensive cell spreading (Supplemental Fig. 4).

Combining HEGF to Hscra and HRGD induced striking different effects. Cells adhering to HEGF/Hscra formed monolayers with multiple cell-to-cell contacts, whereas those seeded on HEGF/HRGD showed a decreased spreading, scattering and partial loss of cytoskeleton organization.

Taken together, these results indicate that the effects induced by pure HEGF on cells scattering, morphology and cytoskeleton (Fig. 2) could be elicited, at lower HEGF concentrations, by the simultaneous

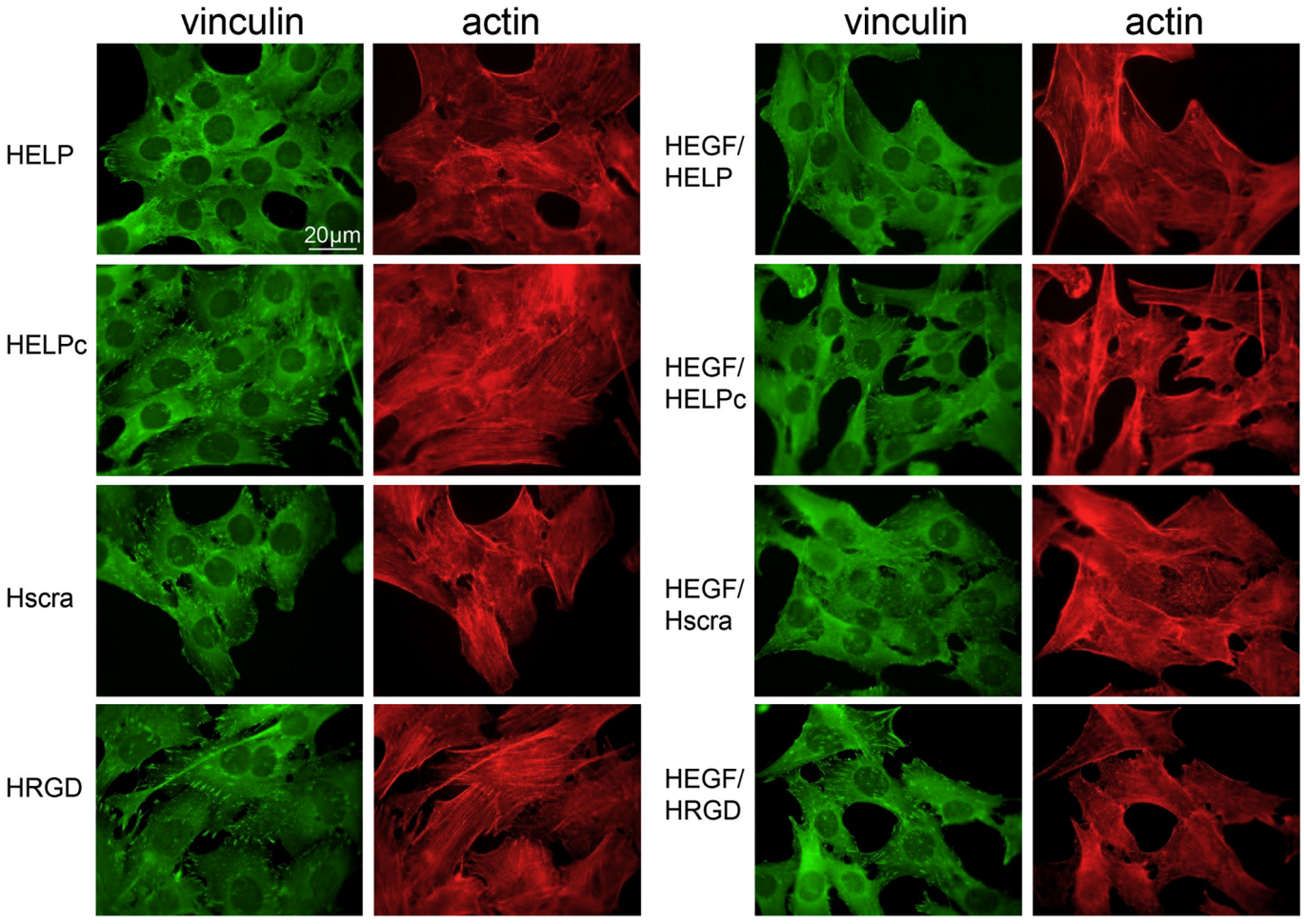

Fig. 3. Effect of mixed substrates on cell adhesion and cytoskeleton. Coverslips were coated with solutions obtained by combining 1 part of HEGF with 4 parts of either HELP, HELPc, Hscra and HRGD. Solutions of pure HELP, HELPc, Hscra and HRGD were employed as reference. C2C12 cells, seeded at a density of 10^4 cells/cm^2 were cultured for 24 h. Immunofluorescence labeled vinculin (green), F-actin was labeled with Atto 594 phalloidin (red). The results are representative of four independent experiments done in triplicate. (n ≥ 450 cells/condition).

presence of RGD sequences (Fig. 3).

In order to evaluate whether the effects of adsorbed HEGF could be mimicked by the free growth factor or by soluble HEGF, cells adhering to either glass or to HELPc coated coverslips were incubated with human EGF (2 nM) or with soluble HEGF at the same concentration (Supplemental Fig. 5). Two different substrates were employed to disclose the possible effects of soluble factors on cell scattering: glass and HELPc. Both soluble EGF and HEGF added to the culture medium failed to induce cell scattering on the two substrates, indicating that growth factor immobilization was required for stimulating the effect.

To gain a deeper insight into the effects of the combined presence of HEGF and RGD, the inhibitors AG82 and herbimycin A were employed on cells seeded on HEGF/HELPc and HEGF/HRGD (Fig. 4). Similarly to what observed with pure HEGF, both AG82 and herbimycin A inhibited scattering, increased cell spreading, focal adhesions assembly and actin cytoskeleton organization. These observations point to the involvement of EGFR and c-src in the signaling pathways activated by cell adhesion to mixed EGF/RGD substrates.

3.3. Cell metabolic activity and proliferation

The involvement of EGF and its receptors in the control of cell survival, proliferation and neoplastic transformation is extensively documented (see [32] for a recent review). In cultured myoblasts, the importance of EGF is recognized during the phase of cell expansion [33], but its effects during myogenic differentiation are still debated [11,34,35].

In a further series of experiments, we evaluated the effects of HEGF on metabolic activity and cell proliferation during both expansion and differentiation phases. Decreasing the serum concentration in myoblasts cultures invariably leads to a time-dependent myogenic differentiation and myotubes formation, as consequence of multiple rounds of cell-to-cell fusion events [36]. In Fig. 5, a scheme resuming the culture protocol employed to test the influence of adhesion substrates on cells metabolic activity is shown. After the initial expansion in Growth Medium (GM), analyzed at 5, 24 and 48 h after seeding, cells were switched to Differentiation Medium (DM) and the culture was carried on for two more days (72 and 96 h after seeding). At these time points, cell-to-cell fusion has not yet started and poly-nucleated cells are not detectable. Metabolic activity was evaluated with the WST-1 assay. In Fig. 5a, the metabolic activity of cells adhering to HELP, HELPc and HEGF is compared. During the first 24 h in culture, the cell activity increased only slightly in each sample. However, 48 h after seeding, the stimulatory effect of both HELPc and HEGF (but not HELP) became evident. At this time point, cultures were shifted to DM. 24 h later, corresponding to 72 h after seeding, cell metabolic activity showed a tendency to decrease in cells cultured on HELP (not significant), whereas a significant increase was evident in cultures on HELPc and HEGF. 96 h after seeding the difference among substrates became remarkable. Cells cultured on HELP maintained a moderate and nearly constant level of activity (2.5 ± 0.6 above the baseline measured 5 h after seeding). In contrast, in cells cultured on HELPc, activity increased

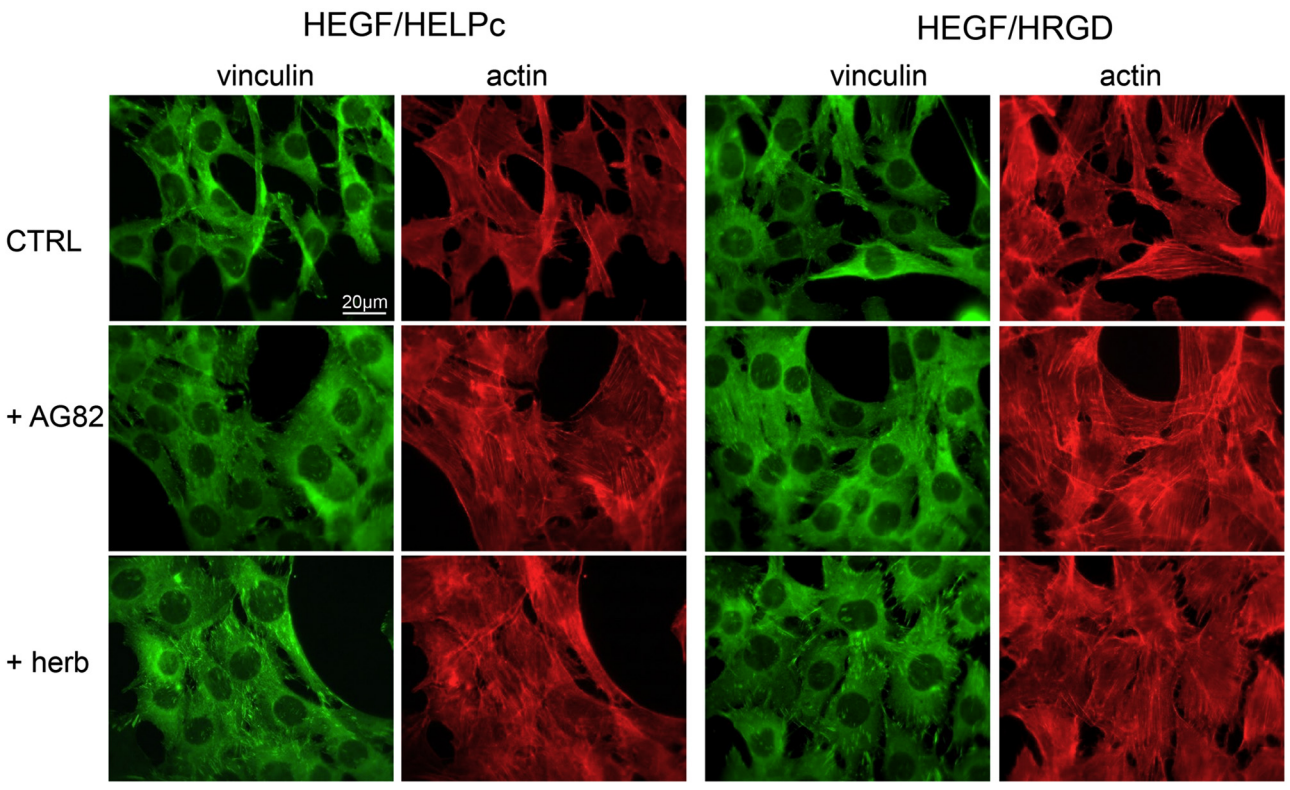

Fig. 4. Effect of tyrosine kinase inhibitors on EGF-induced cell scattering. Coverslips were coated with combined protein solutions obtained by mixing 1 part of HEGF with 4 parts of either HELPc or HRGD. C2C12 cells, seeded at a density of 10^4 cells/cm^2 were cultured for 24 h. Cells were cultured in the absence or in the presence of AG82 (10 μM) or Herbimycin A (0.5 μM). Immunofluorescence labeled vinculin (green), F-actin was labeled with Atto 594 phalloidin (red). The results are representative of three independent experiments done in triplicate. (n ≥ 360 cells/condition).

8.6 ± 0.9 over the baseline and cells cultured on HEGF showed an increase of 13.7 ± 1.8 above the baseline. The presence of AG82 inhibitor largely inhibited the effect of HEGF (Fig. 5b), indicating that activation of the EGFR is required for stimulation.

Next, we systematically compared the metabolic activity of cells seeded on each HELP construct to that induced by the HEGF-combined counterpart. Fig. 5c shows the activity induced by HEGF/HELP compared to that of HELP. The presence of HEGF was sufficient to increase cell activity at each time point, rising up to 10 ± 1.7 above the baseline at 96 h. In Fig. 5d the effects of HELPc and HEGF/HELPc are compared. Although HELPc *per se* stimulated cell activity, the simultaneous presence of HEGF induced a further increase, up to 12.9 ± 2.9 above the baseline. Interesting effects were induced by HRGD and HEGF/HRGD (Fig. 5e). Compared to the other HELPs (excluding HEGF), the HRGD polypeptide stimulated cells activity at a greater extent (11.9 ± 1.9 over the baseline). Moreover, the HEGF/HRGD mix induced the maximal cell stimulation, that reached, at 96 h, the value of 18.4 ± 2.4 above the baseline, thus revealing HEGF/HRGD the mostly effective substrate. Conversely, Hscra showed an effect comparable to that of HELP (3.7 ± 1.2 over the baseline), while the HEGF/Hscra mix led to an increase up to 13.3 ± 2.8.

By comparing the potency of adsorbed and soluble HEGF (Supplemental Fig. 6a), we showed that the adsorbed polypeptide was significantly more effective in stimulating cell metabolic activity. Moreover, when supplemented to the culture medium of cells adhering to uncoated polystyrene, HEGF displayed the same potency of soluble EGF, administered at the same concentration (Supplemental Fig. 6b), indicating that the HELP backbone moiety does not interfere with EGF biological activity.

To correlate metabolic activity to cell proliferation we performed immunofluorescence experiments to detect the expression of Ki-67 [37]. For immunofluorescence, we selected the conditions producing the largest differences in metabolic activities, namely adhesion to HELP, HEGF and HEGF/HRGD (Fig. 5g). The quantitative analysis was performed on cells cultured for 72 and 96 h (Fig. 5h). Compared to cells seeded on HELP, a significant increase of Ki-67 expression characterized cells adhering to HEGF and, at a greater extent, those seeded on the HEGF/HRGD. These results are in good agreement with the WST-1 measures, indicating that, under our experimental conditions, myoblast metabolic activity directly relates to cell proliferation.

Taken together these results indicate that the simultaneous presence of HEGF and RGD sequences stimulated the proliferation of C2C12 myoblasts.

3.4. Cell differentiation

During *in vivo* and *in vitro* myogenesis, activated cells express transcription factors of the myogenic lineage including Myf5, MyoD and myogenin, necessarily required for proliferation of myoblasts and differentiation into myotubes [38]. In the following series of experiments, we evaluated the impact of the adhesion substrates on myogenin expression. Differentiation was triggered by switching cells to DM and lasted 48 h, a time point preceding myotube formation. The results of myogenin immunofluorescence are shown in Fig. 6. In (a) two overlay images show the different level of myogenin expression in cells adhering to HELP and HEGF, respectively. Fig. 6b reports the effect of different coatings on the fraction of myogenin positive cells. Compared to HELP, cell adhesion to HEGF increased the number of myogenin-expressing cells by 2.5 fold, an effect that was inhibited by both AG82 and herbimycin A. The substrates that revealed effective in stimulating myogenin expression were HEGF, HELPc, HEGF/HELPc, HRGD and HEGF/HRGD, while the other coatings (HELP, HEGF/HELP, Hscra and HEGF/Hscra) revealed significantly less active. Thus, the absence of either EGF or RGD in the adhesion substrate significantly impaired the

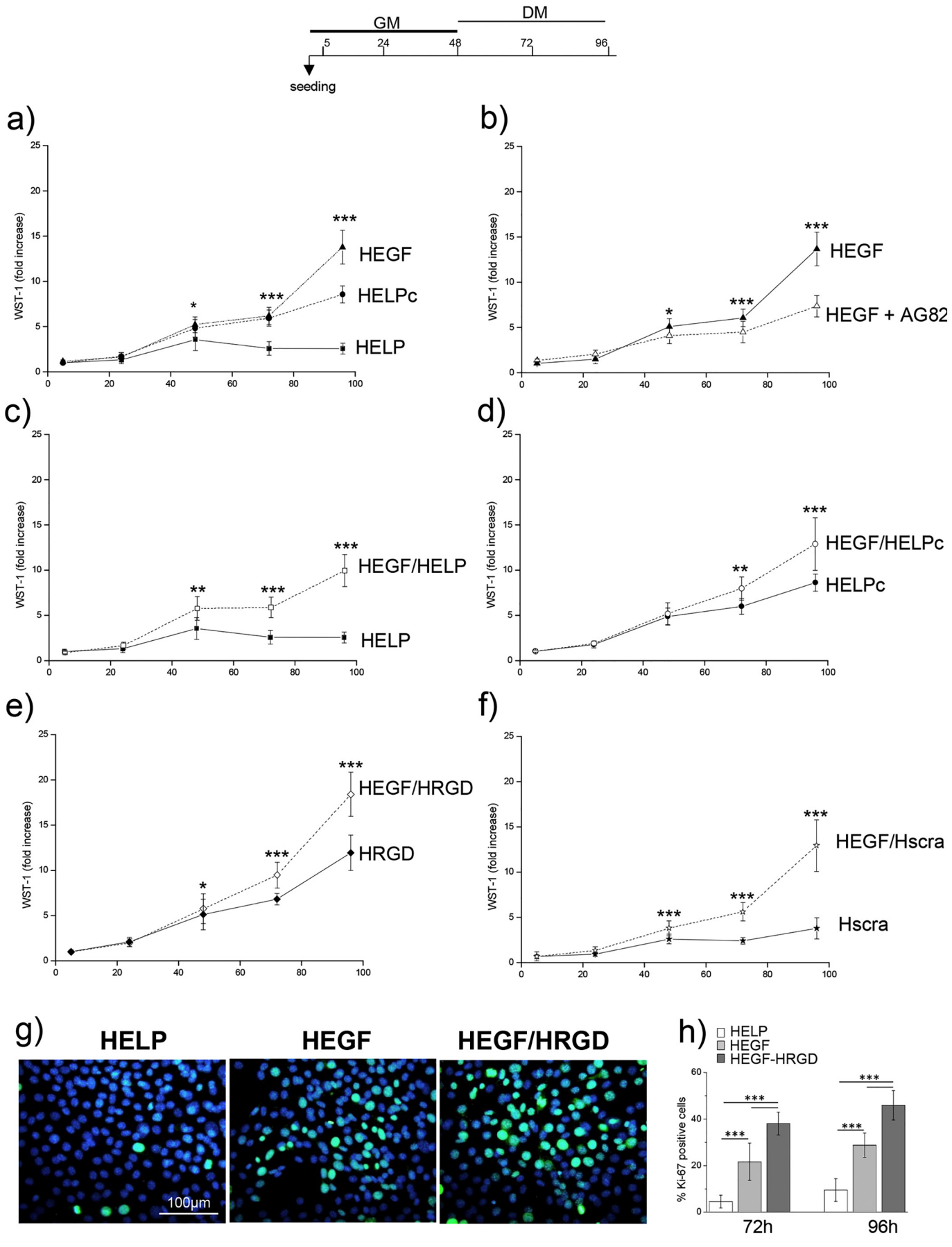

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Fig. 5. Effect of adhesion substrates on cell metabolic activity and proliferation. a–f) Single well bottoms of 96 multiwells plates were coated with solutions of each indicated polypeptide. C2C12 cells were seeded at a density of 5×10^3 cells/well and cultured as schematized in the diagram (top). a) Comparison of the metabolic activity induced by cell adhesion to HELP, HELPc and HEGF coated substrates. b) Effect of the EGF receptor inhibitor AG82 (10 μM) on the metabolic activity induced by adhesion to HEGF. In panels c-f the metabolic activity of each adhesion substrate is compared to that induced by the HEGF-mixed counterpart. c) Metabolic activity of HELP compared to that of HEGF/HELP. d) Metabolic activity of HELPc and HEGF/HELPc. e) Metabolic activity of HRGD and HEGF/HRGD. f) Metabolic activity of Hscra and HEGF/Hscra. Results of three independent experiments done in quadruplicate. g–h) C2C12 proliferation assessed by Ki-67 immunofluorescence. Cells were seeded at a density of 5×10^3 cells/cm^2 onto coverslips coated with HELP, HEGF and a combined substrate HEGF/HRGD obtained by mixing 1 part of HEGF and 4 parts of HRGD. At 48 h cells were induced to differentiate for one or two days (72 h and 96 h after seeding). Samples were then fixed and processed for immunofluorescence. g) Overlay images of Ki-67 expressing cells adhering to HELP, HEGF and HELP/HEGF. Immunostaining decorated Ki-67 (green), DAPI counterstained nuclei (blue). h) Fraction (%) of Ki-67 expressing cells at 72 h and 96 h in culture. Results from two independent experiments done in triplicate. (n ≥ 2400 cells/condition). *p < 0.05; **p < 0.01; ***p < 0.001.

increase of myogenin expression in response to differentiative cues.

During the later stages of development, the newly formed multinucleated myotubes start to express other differentiation markers, such as the muscle-type Myosin Heavy Chain (MHC), detected also in differentiated C2C12 myotubes [39]. Hence, we investigated the expression of MHC at 4 days of differentiation. In Fig. 6c, the overlay images of MHC-expressing myotubes and DAPI-stained nuclei are shown. Remarkable differences are evident in the myogenic differentiation among the cultures on different substrates. HELP and Hscra appeared as the less permissive substrates for myotube development, while HEGF, HELPc, HRGD, HEGF/HELPc and, most of all, HEGF/HRGD, gave rise to cultures in which myotubes revealed more mature and enlarged, enclosing many nuclei. Particularly, myotubes developed on HEGF/HRGD appeared larger and branched, indicating an advanced developmental

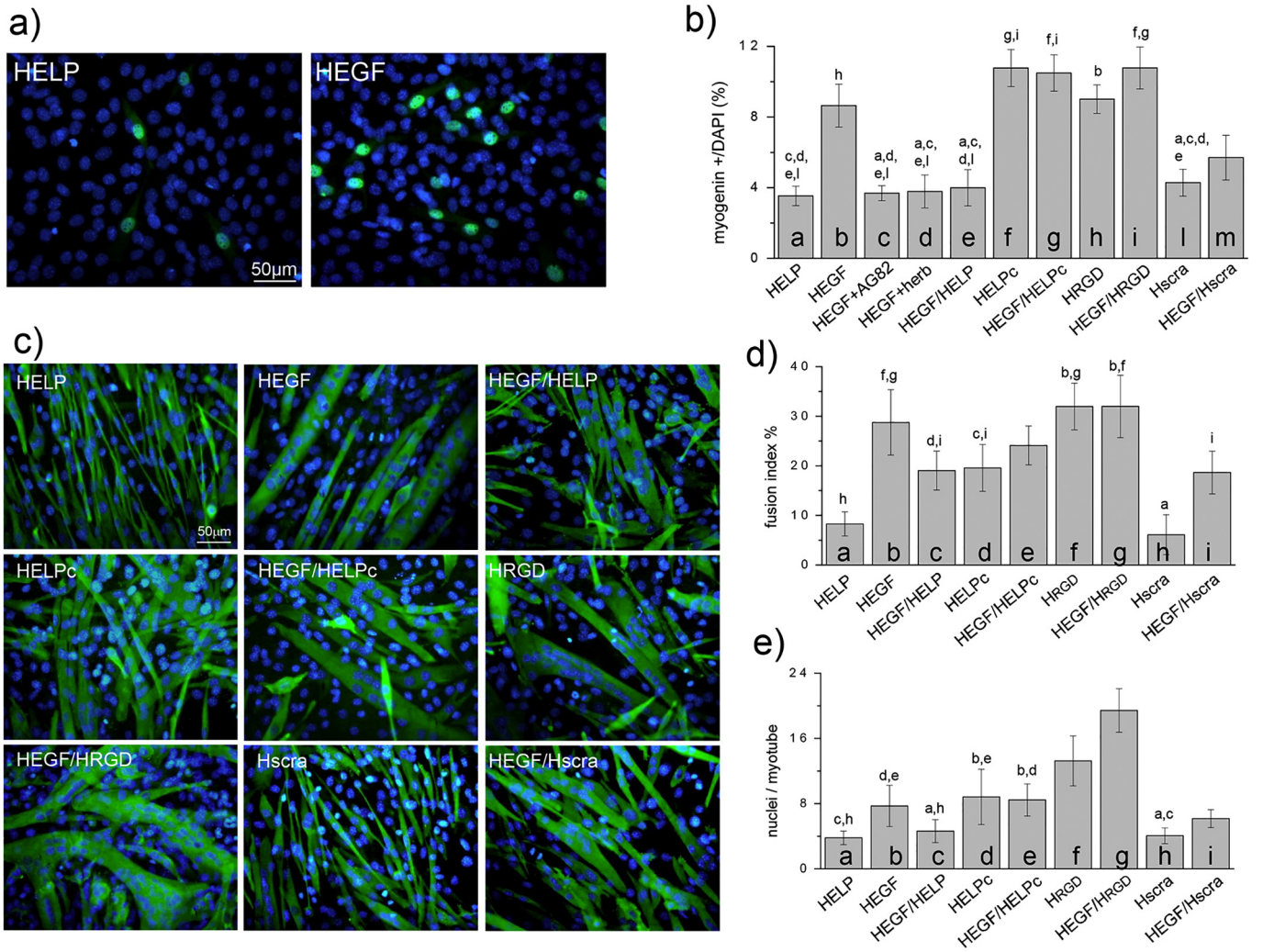

Fig. 6. Effect of adhesion substrates on C2C12 differentiation. Cells were seeded at a density of 5×10^3 cells/cm^2. Two days later, when cell confluence reached 75%, GM was replaced with DM. For myogenin detection, culture proceeded for two more days, after which samples were fixed and processed for immunostaining. a) Overlay images of myogenin expressing cells seeded on HELP and on HEGF. Immunofluorescence labeled myogenin (green), DAPI counterstained nuclei (blue). b) Fraction of myogenin expressing cells. Results of three independent experiments done in triplicate (n ≥ 2500 cells/condition). For Myosin Heavy Chain detection (c–e), culture in DM proceeded for four days. c) Overlay images of MHC expressing cells. Immunofluorescence labeled Myosin Heavy Chain (MHC, green), DAPI counterstained nuclei (blue). d) Fusion index analysis of myotubes. e) Comparison of the number of nuclei present in MHC-positive myotubes. Results from six independent experiments done in triplicate. (n ≥ 5500 cells/condition). For statistical comparisons, each data set was tested *vs* the remaining data sets. Letters above each column represent data sets that were found to be not significantly different with respect to that column.

stage. To quantitatively compare the differences observed in immunofluorescence, we calculated the fusion index and the average number of nuclei in MHC-positive myotubes (Fig. 6d–e). The fusion index accounts for the tendency of myoblasts to withdraw the cell cycle and fuse with neighboring cells, while the nuclei number highlights the tendency of myotubes to undergo multiple fusion events. The fusion index analysis (Fig. 6d) confirmed the qualitative indications of Fig. 6c, pointing to HEGF, HRGD and HEGF/HRGD as the most favorable substrates for myotubes development, while HELP and Hscra resulted the least effective. Once more, an intriguing difference was detected between HELPc and HRGD, with the latter substantially more efficient in promoting myotubes maturation. The analysis of nuclei number (Fig. 6e), besides confirming the results of fusion index analysis, disclosed further important differences among the substrates. The stimulatory effect of HRGD and, especially, that of HEGF/HRGD coating indicates that these substrates stimulate multiple rounds of cell-to-cell fusion, leading to the development of thoroughly nucleated syncytia. To ascertain whether HEGF-induced cell differentiation effects depended on its anchoring to the adhesion substrate, cells adhering to either glass or to HELPc coated coverslips were cultured and differentiated in the presence of soluble HEGF (2 nM). As already reported [15], adhesion to HELPc markedly increase myogenesis, compared to the glass control. However, cell supplemented with soluble HEGF in the culture medium did not show an increase in myogenin expression and myotube formation whatever the substrate (Supplemental Fig. 7).

3.5. *HEGF-dependent activation of satellite cells*

The use of the immortal C2C12 cell line allowed us to test several substrates, experimental conditions and different properties. However, although these cells maintain *in vitro* most of the properties of native myoblasts, including the ability to undergo myogenic differentiation, we cannot exclude that the molecular events leading to immortalization could have partially altered their sensitivity to the extracellular environment. The remarkable responses induced by HEGF in these cells prompted us to test its effects on primary cultures of satellite cells. To this purpose, we used satellite cells derived from cultures of isolated *flexor digitorum brevis* (FDB) muscle fibers [40,41]. With this method, after skeletal muscle cell plating, satellite cells start to detach from skeletal fiber surface, migrate and divide; by few days (4–6), they begin to fuse into myotubes. With respect to other methods [42], this procedure maintains satellite cells beneath the basal lamina, thus allowing to analyze their behavior starting from the early phases of cell activation. Moreover, the degree of contamination by other cells, although not totally avoided, is considerably decreased [41]. The adhesion of skeletal myofibers to glass coverslips, however, necessarily requires several ECM components, among which laminin, type IV collagen, heparan sulfate and entactin [43,44], which are usually supplied by coating the surfaces with Matrigel, a mixture of ECM proteins which provides a 2D protein layer adsorbed to glass coverslips [45]. Consequently, to test the effects of HEGF on satellite cells, we plated myofibers on combined substrates composed of HEGF and Matrigel. We compared the effects of HEGF to those induced by Matrigel coated support and to those generated by a mixed HELP/Matrigel coating. In a first series of experiments, we assayed the presence of the paired box transcription factor 7 (Pax7), a satellite cell marker whose expression is required for acquisition of muscle commitment [46]. We compared Pax7 expression in mononucleated cells present in cultures 72 h after myofibers plating (Fig. 7a–c). In Fig. 7a the immunofluorescence images show that adhesion to HEGF/Matrigel was associated to an increased number of $Pax7^+$ cells surrounding myofibers, compared to control. Fig. 7b shows the number of Pax7-expressing cells per area and in Fig. 7c the percentage of $Pax7^+$ nuclei is reported. The results indicate a marked stimulatory effect of HEGF/Matrigel on the amount of $Pax7^+$ cells present in the myofibers cultures, both in terms of absolute values and in terms of culture enrichment in satellite cells. Intriguingly, although cell adhesion to HELP/Matrigel, compared to control Matrigel, increased the number of $Pax7^+$ cells per area, the fraction of Pax7-expressing nuclei is similar, suggesting that HELP/Matrigel, in contrast to HEGF/Matrigel increased cell proliferation/survival of both satellite and other, contaminating, cell types.

We next examined myogenin expression 72 h after seeding. Indeed, although myogenin is considered a late myogenic marker, transitional stages of newly differentiated myoblasts often co-express both Pax7, MyoD and myogenin [47]. In Fig. 7d the immunofluorescence images show an increase of myogenin-expressing nuclei in cell adhering to HEGF/Matrigel coatings, compared to controls. Similarly to what found for Pax7, HEGF/Matrigel induced an increase of both the total number $myogenin^+$ cells (Fig. 7e) and the fraction of nuclei expressing myogenin (Fig. 7f).

Taken together, these results demonstrated that adhesion of myofibers to substrate coatings containing HEGF increased satellite cells activation without hampering their myogenic potential.

4. Discussion

The presence of growth factors is crucial for tissue survival and regeneration [48]. Immobilization of growth factors ensures their stability and persistence, providing cells with a constant level of stimulation, ultimately increasing their efficacy [49]. Several members of the EGF-family of growth factors bind to EGFR, including Transforming Growth Factor, amphiregulin, betacellulin, epiregulin, and heparin-binding EGF-like growth factor [50]. Intriguingly, some of them, or their precursors, activate cognate receptors on adjacent cells and communicate by juxtacrine stimulation. However, due to differences in half-life, spatial control and desensitization mechanisms, the cellular responses to immobilized growth factors are not easily predictable. A remarkable example is given by PC12 cells that, when stimulated with soluble EGF, proliferated and maintained a chromaffin-like morphology, while, when seeded on immobilized EGF, arrested the cell cycle, emitted neurites and undergo neural differentiation similar to that induced by diffusible NGF [51]. This apparent paradox has been explained on the basis that immobilized EGF transduced its signal for longer time than its soluble form. Indeed, immobilized EGF continuously activated mitogen-activated protein kinase (MAPK) whereas the MAPK activation induced by soluble EGF decreased rapidly with time [51].

When C2C12 myoblasts adhere to HELP-based coatings, they assume a morphology and a cytoarchitecture that depends on the polypeptide sequence ([15], present work). Cell adhesion to HEGF induced striking modifications of cell morphology, with cell scattering, cytoskeleton rearrangement and focal adhesion disassembly. Increased cell scattering is one of the most consistent effects induced by EGF in many cell types [52] which, acting through inhibition of adhering junctions and disruption of cell-to-cell contacts (see, among others, [53,54]), increases *in vitro* cell motility [55,56]. Our data showed that the presence of RGD motifs in the adhesion substrate reduced the amount of HEGF required for inducing cell scattering, suggesting that these sequences supply permissive signals capable to lower the threshold of EGF-dependent responses. These results highlight the importance of the cross-talk between EGF and RGD signaling in dictating myoblasts responses to extracellular cues. Accumulating evidence indicate that the reactions triggered by EGFR activation mutually and synergistically interact with those induced by integrins engagement, thus crucially linking soluble and immobilized signals in the shaping of the ultimate cell fate [30,31,57]. The simultaneous presence of EGF and RGD, besides affecting cell adhesion and cytoskeleton, stimulated cell proliferation, metabolic activity and myogenic differentiation of C2C12 cells, emphasizing the impact that the cross-talk exerts on skeletal muscle development. Moreover, the fact that the scattering effect induced by both HEGF and HEGF/RGD mixed substrates was inhibited herbimycin A, suggests that c-src activation, by bi-directionally linking

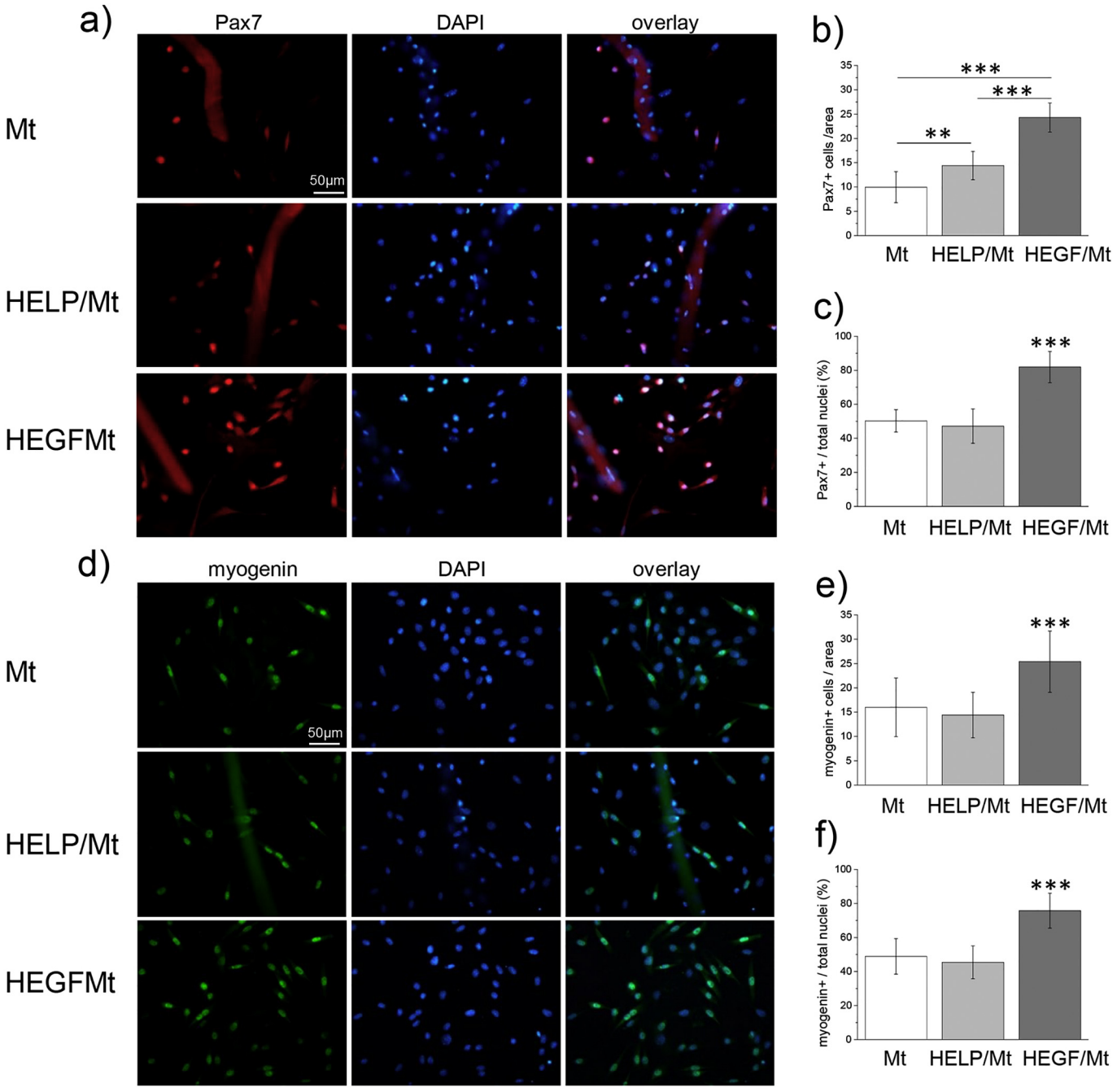

Fig. 7. Effect of HEGF on satellite cells activation and myogenesis. Isolated FDB myofibres were plated on coverslips coated as follows: Mt.: Matrigel solution (control). HELP/Mt.: HELP and Matrigel mixed solution. HEGF/Mt.: HEGF and Matrigel mixed solution. Cells were cultured for 72 h. a) Immunofluorescence labeled Pax7 (red), DAPI stained nuclei (blue). b) Number of Pax7-expressing cells per microscopic field. c) Percentage of Pax7-expressing cells. The total number of nuclei and of Pax7-positive mononucleated cells was counted in at least 60 random fields for each condition (n ≥ 2000 cells/condition). Results from two independent experiments done in triplicate. d) Immunofluorescence labeled myogenin (green), DAPI counterstained nuclei (blue). e) Number of myogenin-expressing cells per microscopic field. f) Percentage of myogenin-expressing cells. The total number of nuclei and of myogenin-positive mononucleated cells was counted in at least 75 random fields for each condition (n ≥ 2600 cells/condition). Results are from two independent experiments done in triplicate. **$p < 0.01$; ***$p < 0.001$.

EGF- and integrin- dependent signaling [58], dictates the flow of reactions activated downstream of adhesion and growth factor receptors [59]. Most importantly, the effects of immobilized HEGF on cell scattering and myogenic differentiation could not be induced by the soluble EGF (scattering) and HEGF (scattering and differentiation) supplemented to the culture medium, while cell proliferation and metabolic activity were stimulated by both compounds, though at a lesser extent than immobilized HEGF. Taken together, these data suggest that the effects induced by cells attachment to HEGF coating are linked to the persistent activation of EGFR and/or to the spatial control provided by agonist immobilization.

The effects of EGFR activation on cultured myoblasts have been disputed. Downregulation of EGFR has been proposed to be required for differentiation [34,35]; however, the protective effect of membrane-bound HB-EGF against the pro-apoptotic signals that cells experience during differentiation [11], suggests that persistent receptor activation could contribute to myogenesis.

In our work, HEGF and HEGF/RGD-containing coatings increased

cell proliferation, in particular when the concentration of serum growth factors was drastically reduced. This suggests that the sustained activation of EGFR decreases the requirement for diffusible mitogens. The proliferative effect of adhesion substrates was evaluated before the beginning of cell-to-cell fusion and myotubes formation. At this time, however, myogenin was already expressed by 4–10% of cells, indicating that proliferation did not prevent myogenesis. Indeed, as culture proceeded, multinucleated myotubes partially replaced myoblasts and Myosin Heavy Chain became detectable. In the differentiation assays, the effect of substrates was remarkable, pointing to both HEGF and HRGD, or their mixture, as the adhesion proteins that most efficiently stimulated myogenic differentiation. In particular, adhesion to HEGF/HRGD induced cells to undergo extensive fusion, giving rise to thick, heavily branched, multinucleated myotubes. The results suggest that EGFR activation and, most of all, the cross-talk between EGFR- and integrin-dependent signaling conveys the optimal information for myogenesis to occur.

Intriguingly, we systematically observed a different potency of HELPc and its scrambled counterpart HRGD in promoting both proliferation and differentiation. Since the two polypeptides maintain the RGD motifs in the same position, our results indicate that the aminoacid sequences surrounding RGD in type IV collagen interfere with the signaling pathways activated by EGF and adhesion signals, thus suggesting a modulatory role for this sequence in the native protein.

Adult skeletal muscle growth, repair, and regeneration depend on the activation of satellite cells, the muscle stem cells resident beneath the basal lamina enwrapping myofibers. In response to appropriate stimuli, satellite cells exit from quiescence, proliferate, migrate and fuse to form new muscle fibers, recapitulating muscle development during embryogenesis (see [60] for an extensive review). Given their capability to maintain the intrinsic potential to regenerate skeletal muscle, satellite cells are considered among the most promising candidates for cell transplantation and cell-based regeneration therapies [61]. During activation from quiescence, satellite cells express the EGFR isoforms erbB1 and erbB2, together with the other members of the receptor superfamily [62]. Collectively, these receptors have been proposed to protect satellite cells from the pro-apoptotic signals they experience upon activation. Our results are in agreement with such hypothesis, given that adhesion to HEGF-enriched Matrigel increased both Pax7- and myogenin- expressing mononucleated satellite cells, thus proving that the myogenic effect of HEGF is experienced also by satellite cells obtained from isolated myofibers. The last evidence could be important in the perspective of tissue regeneration strategies. Indeed, a major hindrance in muscle cell therapy is the scarcity of progenitor cells for successful transplantation. As such, an *in vitro* expansion step, capable of preserving cell myogenic potential, represents one of the aims actively pursued [61,63,64]. In this view, the stimulatory effect of HEGF on satellite cells appears promising and inspiring of future studies intended to test the suitability of HEGF-based substrates for the *in vitro* expansion of satellite cells.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

The authors declare no conflicts of interests. The authors thank Gigi Nocera for preparing isolated myofiber cultures. This work was supported in part by Università degli Studi di Trieste (Finanziamento di Ateneo per progetti di ricerca scientifica - FRA2013) and by Beneficentia Stiftung (BEN 2014/125), Vaduz, Lichtenstein. We acknowledge the financial support from Central European Research Infrastructure Consortium CERIC-ERIC, proposal n. 20172010.

The funding sources had no involvement in the study design, collection and interpretation of data.

Author contributions

PDA AB proposed the experimental basis and designed the experiments; PDA AB MS KV performed the experiments; PDA AB PL KV analyzed the data and interpreted the results; PDA AB drafted the manuscript; PL MS KV made edits for the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online.

References

[1] M. Kanagawa, T. Toda, The genetic and molecular basis of muscular dystrophy: roles of cell-matrix linkage in the pathogenesis, J. Hum. Genet. 51 (2006) 915–926.
[2] D.K. Lund, D.D. Cornelison, Enter the matrix: shape, signal and superhighway, FEBS J. 280 (2013) 4089–4099.
[3] J. Massagué, A. Pandiella, Membrane-anchored growth factors, Annu. Rev. Biochem. 62 (1993) 515–541.
[4] A. Sahni, T. Odrljin, C.W. Francis, Binding of basic fibroblast growth factor to fibrinogen and fibrin, J. Biol. Chem. 273 (1998) 7554–7559.
[5] R.L. Driessen, H.M. Johnston, S.K. Nilsson, Membrane-bound stem cell factor is a key regulator in the initial lodgment of stem cells within the endosteal marrow region, Exp. Hematol. 31 (2003) 1284–1291.
[6] S. Barrientos, O. Stojadinovic, M.S. Golinko, H. Brem, M. Tomic-Canic, Growth factors and cytokines in wound healing, Wound Repair Regen. 16 (2008) 585–601.
[7] G. Panayotou, P. End, M. Aumailley, R. Timpl, J. Engel, Domains of laminin with growth-factor activity, Cell 56 (1989) 93–101.
[8] W. Hoch, Molecular dissection of neuromuscular junction formation, Trends Neurosci. 26 (2003) 335–337.
[9] T. Cornish, J. Chi, S. Johnson, Y. Lu, J.T. Campanelli, Globular domains of agrin are functional units that collaborate to induce acetylcholine receptor clustering, J. Cell Sci. 112 (1999) 1213–1223.
[10] R.W. Burgess, D.K. Dickman, L. Nunez, D.J. Glass, J.R. Sanes, Mapping sites responsible for interactions of agrin with neurons, J. Neurochem. 83 (2002) 271–284.
[11] M. Horikawa, S. Higashiyama, S. Nomura, Y. Kitamura, M. Ishikawa, N. Taniguchi, Upregulation of endogenous heparin-binding EGF-like growth factor and its role as a survival factor in skeletal myotubes, FEBS Lett. 459 (1999) 100–104.
[12] A. Bandiera, A. Taglienti, F. Micali, B. Pani, M. Tamaro, V. Crescenzi, G. Manzini, Expression and characterization of human-elastin-repeat-based temperature responsive protein polymers for biotechnological purposes, Biotechnol. Appl. Biochem. 42 (2005) 247–256.
[13] A. Bandiera, Assembly and optimization of expression of synthetic genes derived from the human elastin repeated motif, Prep. Biochem. Biotechnol. 40 (2010) 198–212.
[14] G. Ciofani, G.G. Genchi, I. Liakos, A. Athanassiou, V. Mattoli, A. Bandiera, Human recombinant elastin-like protein coatings for muscle cell proliferation and differentiation, Acta Biomater. 9 (2013) 5111–5121.
[15] P. D'Andrea, D. Scaini, L. Ulloa Severino, V. Borelli, S. Passamonti, P. Lorenzon, A. Bandiera, In vitro Myogenesis induced by human recombinant elastin-like proteins, Biomaterials 67 (2015) 240–253.
[16] P. D'Andrea, D. Civita, M. Cok, L. Ulloa Severino, F. Vita, D. Scaini, L. Casalis, P. Lorenzon, I. Donati, A. Bandiera, Myoblasts adhesion, proliferation and differentiation on Human Elastin-Like Polypeptide (HELP) hydrogels, J. Appl. Biomater. Funct. Mater. 15 (2017) e43–e53.
[17] P. Stothard, The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences, BioTechniques 28 (2000) 1102–1104.
[18] L. Corich, M. Busetti, V. Petix, S. Passamonti, A. Bandiera, Evaluation of a biomimetic 3D substrate based on the Human Elastin-like Polypeptides (HELPs) model system for elastolytic activity detection, J. Biotechnol. 255 (2017) 57–65.
[19] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH image to ImageJ: 25 years of image analysis, Nat. Methods 9 (2012) 671–675.
[20] A. Bekoff, W. Betz, Properties of isolated adult rat muscle fibers maintained in tissue culture, J. Physiol. 271 (1977) 537–547.
[21] E. Vanea, V. Simon, XPS study of protein adsorption onto nanocrystalline aluminosilscate microparticles, Appl. Surf. Sci. 257 (2011) 2346–2352.
[22] V. Secchi, S. Franchi, M. Fioramonti, G. Polzonetti, G. Iucci, B. Bochicchio, Ch. Battochi, Nanofibres of human Tropoelastin-inspired peptides: structural characterization and biological properties, Mater. Sci. Eng. C 77 (2017) 927–934.
[23] S.S. Jedlicka, J.L. Rickus, D.Y. Zemlyanov, Surface analysis by X-ray photoelectron spectroscopy of sol-gel silica modified with covalently bound peptides, J. Phys. Chem. 111 (2007) 11850–11857.
[24] S. Franchi, Ch. Battocchio, M. Galluzi, E. Navisse, A. Zamuner, M. Dettin, G. Iucci, Self-assembling peptides hydrogels immobilized on silicon surfaces, Mater. Sci. Eng. C 69 (2016) 200–207.
[25] J.H. Moon, W. Bahng, I.H. Kang, S.C. Kim, M.G. Na, N.K. Kim, Improved 4H-SiC

metal oxide semiconductor interface produced by using an oxidized SiN gate oxide that had undergone post-oxidation annealing, J. Korean Phys. Soc. 64 (2014) 1363–1369.
[26] A. Levitzki, E. Mishani, Tyrphostins and other tyrosine kinase inhibitors, Annu. Rev. Biochem. 75 (2006) 93–109.
[27] H. Fukazawa, P.M. Li, C. Yamamoto, Y. Murakami, S. Mizuno, Y. Uehara, Specific inhibition of cytoplasmic protein tyrosine kinases by herbimycin A in vitro, Biochem. Pharmacol. 42 (1991) 1661–1671.
[28] A.K. Howe, A.E. Aplin, R.L. Juliano, Anchorage-dependent ERK signaling–mechanisms and consequences, Curr. Opin. Genet. Dev. 12 (2002) 30–35.
[29] L. Moro, M. Venturino, C. Bozzo, L. Silengo, F. Altruda, L. Beguinot, G. Tarone, P. Defilippi, Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival, EMBO J. 17 (1999) 6622–6632.
[30] H.M. Bill, B. Knudsen, S.L. Moores, S.K. Muthuswamy, V.R. Rao, J.S. Brugge, C.K. Miranti, Epidermal growth factor receptor-dependent regulation of integrin-mediated signaling and cell cycle entry in epithelial cells, Mol. Cell. Biol. 24 (2004) 8586–8599.
[31] P. Eberwein, D. Laird, S. Schulz, T. Reinhard, T. Steinberg, TomakidiP. Modulation of focal adhesion constituents and their down-stream events by EGF: on the cross-talk of integrins and growth factor receptors, Biochim. Biophys. Acta 1853 (2015) 2183–2198.
[32] M. Di Domenico, A. Giordano, Signal transduction growth factors: the effective governance of transcription and cellular adhesion in cancer invasion, Oncotarget 8 (2017) 36869–36884.
[33] D. Jarocha, K. Stangel-Wojcikiewicz, A. Basta, M. Majka, Efficient myoblast expansion for regenerative medicine use, Int. J. Mol. Med. 34 (2014) 83–91.
[34] B.B. Olwin, S.D. Hauschka, Cell surface fibroblast growth factor and epidermal growth factor receptors are permanently lost during skeletal muscle terminal differentiation in culture, J. Cell Biol. 107 (1988) 761–769.
[35] M.C. Leroy, J. Perroud, B. Darbellay, L. Bernheim, S. Konig, Epidermal growth factor receptor down-regulation triggers human myoblast differentiation, PLoS One 8 (2013) e71770.
[36] D. Yaffe, O. Saxel, Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle, Nature 270 (1977) 725–727.
[37] T. Scholzen, J. Gerdes, The Ki-67 protein: from the known and the unknown, J. Cell. Physiol. 182 (2000) 311–322.
[38] C.F. Bentzinger, Y.X. Wang, M.A. Rudnicki, Building muscle: molecular regulation of myogenesis, Cold Spring Harb. Perspect. Biol. 4 (2012) a008342.
[39] J.B. Miller, Myogenic programs of mouse muscle cell lines: expression of myosin heavy chain isoforms, MyoD1, and myogenin, 111 (1990) 1149–1159.
[40] F. Grohovaz, P. Lorenzon, F. Ruzzier, R. Zorec, Properties of acetylcholine receptors in adult rat skeletal muscle fibers in culture, J. Membr. Biol. 136 (1993) 31–42.
[41] J.D. Rosenblatt, A.I. Lunt, D.J. Parry, T.A. Partridge, Culturing satellite cells from living single muscle fiber explants, In Vitro Cell. Dev. Biol. Anim. 31 (1995) 773–779.
[42] M.E. Danoviz, Z. Yablonka-Reuveni, Skeletal muscle satellite cells: background and methods for isolation and analysis in a primary culture system, Methods Mol. Biol. 798 (2012) 21–52.
[43] V.L. Funanage, S.M. Smith, M.A. Minnich, Entactin promotes adhesion and long-term maintenance of cultured regenerated skeletal myotubes, J. Cell. Physiol. 150 (1992) 251–257.
[44] K.J. Wilschut, H.P. Haagsman, B.A. Roelen, Extracellular matrix components direct porcine muscle stem cell behavior, Exp. Cell Res. 316 (2010) 341–352.
[45] G. Shefer, Z. Yablonka-Reuveni, Isolation and culture of skeletal muscle myofibers as a means to analyze satellite cells, Methods Mol. Biol. 290 (2005) 281–304.
[46] P.S. Zammit, F. Relaix, Y. Nagata, A.P. Ruiz, C.A. Collins, T.A. Partridge, J.R. Beauchamp, Pax7 and myogenic progression in skeletal muscle satellite cells, J. Cell Sci. 119 (2006) 1824–1832.
[47] Z. Yablonka-Reuveni, K. Day, A. Vine, G. Shefer, Defining the transcriptional signature of skeletal muscle stem cells, J. Anim. Sci. 86 (2008) E207–E216.
[48] B.C. Syverud, K.W. Van Dusen, L.M. Larkin, Growth factors for skeletal muscle tissue engineering, Cells Tissues Organs 202 (2016) 169–179.
[49] K.S. Masters, Covalent growth factor immobilization strategies for tissue repair and regeneration, Macromol. Biosci. 11 (2011) 1149–1163.
[50] D.J. Riese 2nd, D.F. Stern, Specificity within the EGF family/ErbB receptor family signaling network, BioEssays 20 (1998) 41–48.
[51] Y. Ito, G. Chen, Y. Imanishi, T. Morooka, E. Nishida, Y. Okabayashi, M. Kasuga, Differential control of cellular gene expression by diffusible and non-diffusible EGF, J. Biochem. 129 (2001) 733–737.
[52] P. Chen, K. Gupta, A. Wells, Cell movement elicited by epidermal growth factor receptor requires kinase and autophosphorylation but is separable from mitogenesis, J. Cell Biol. 124 (1994) 547–555.
[53] Z. Lu, S. Ghosh, Z. Wang, T. Hunter, Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion, Cancer Cell 4 (2003) 499–515.
[54] R. Singh, P. Lei, S.T. Andreadis, PKC-delta binds to E-cadherin and mediates EGF-induced cell scattering, Exp. Cell Res. 315 (2009) 2899–2913.
[55] Y. Barrandon, H. Green, Cell migration is essential for sustained growth of keratinocyte colonies: the roles of transforming growth factor-alpha and epidermal growth factor, Cell 50 (1987) 1131–1137.
[56] K. Westermark, M. Nilsson, T. Ebcndal, B. Westermark, Thyrocyte migration and histiotypic follicle regeneration are promoted by epidermal growth factor in primary culture of thyroid follicles in collagen gel, Endocrinology 129 (1991) 2180–2186.
[57] R.S. Ross, Molecular and mechanical synergy: cross-talk between integrins and growth factor receptors, Cardiovasc. Res. 63 (2004) 381–390.
[58] K. Sato, Cellular functions regulated by phosphorylation of EGFR on Tyr845, Int. J. Mol. Sci. 14 (2013) 10761–10790.
[59] S. Huveneers, E.H. Danen, Adhesion signaling - crosstalk between integrins, Src and Rho, J. Cell Sci. 122 (2009) 1059–1069.
[60] J.E. Anderson, The satellite cell as a companion in skeletal muscle plasticity: currency, conveyance, clue, connector and colander, J. Exp. Biol. 209 (2007) 2276–2292.
[61] T.H. Qazi, D.J. Mooney, M. Pumberger, S. Geissler, G.N. Duda, Biomaterials based strategies for skeletal muscle tissue engineering: existing technologies and future trends, Biomaterials 53 (2015) 502–521.
[62] J.P. Golding, E. Calderbank, T.A. Partridge, J.R. Beauchamp, Skeletal muscle stem cells express anti-apoptotic ErbB receptors during activation from quiescence, Exp. Cell Res. 313 (2007) 341–356.
[63] S. Ostrovidov, X. Shi, R.B. Sadeghian, S. Salehi, T. Fujie, H. Bae, M. Ramalingam, A. Khademhosseini, Stem cell differentiation toward the myogenic lineage for muscle tissue regeneration: a focus on muscular dystrophy, Stem Cell Rev. 11 (2015) 866–884.
[64] W.M. Han, Y.C. Jang, A.J. García, Engineered matrices for skeletal muscle satellite cell engraftment and function, Matrix Biol. 60-61 (2017) 96–109.