Paracrine signalling events in embryonic stem cell renewal mediated by affinity targeted nanoparticles

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Bruna Corradetti [a,b], Paz Freile [a], Steve Pells [a], Pierre Bagnaninchi [a], Jason Park [c], Tarek M. Fahmy [c,d], Paul A. de Sousa [a,*]

[a] Scottish Centre for Regenerative Medicine, University of Edinburgh, Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK
[b] Department of Life and Environmental Sciences, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy
[c] Department of Biomedical Engineering, Yale University, Malone Engineering Centre, 55 Prospect St., New Haven, CT 06511, USA
[d] Department of Chemical and Environmental Engineering, Yale University, USA

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ABSTRACT

Stem cell growth and differentiation is controlled by intrinsic and extrinsic factors. The latter includes growth factors, which are conventionally supplied in vitro in media exchanged daily. Here, we illustrate the use of affinity targeted biodegradable nanoparticles to mediate paracrine stimulation as an alternative approach to sustain the growth and pluripotency of mouse embryonic stem cells. Leukaemia Inhibitory Factor (LIF) was encapsulated in biodegradable nanoparticles and targeted to the cell surface using an antibody to the oligosaccharide antigen SSEA-1. Sustained release of LIF from nanoparticles composed of a solid Poly(lactide-co-glycolic acid) polyester or a hydrogel-based liposomal system, we term Nanolipogel, replenished once after each cell passage, proved as effective as daily replenishment with soluble LIF for maintenance of pluripotency after 5 passages using 10^4-fold less LIF. Our study constitutes an alternative paradigm for stem cell culture, providing dynamic microenvironmental control of extrinsic bioactive factors benefiting stem cell manufacturing.

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1. Introduction

In vitro systems supporting stem cell renewal and differentiation are challenged by uncontrolled cell behaviour and death affecting their efficiency. This undermines their utility in research, and in translational applications such as the manufacture of specific cell populations for screening or therapeutic purposes. Irrespective of their scale or nature, cell culture systems conventionally supply exogenous bioactive factors by solubilisation in culture medium. This becomes costly during prolonged culture over weeks and months, especially when there is a requirement for complex cocktails of protein factors to expand or direct differentiation of cells to a specific endpoint [1,2], as for example during directed differentiation of human embryonic stem cells (hESC) populations to neuronal subtypes or even expansion of mesenchymal [3] or organ specific stem cell populations for autologous or allogeneic transplantation [4–6]. Exogenously-supplied factors can also support the viability or growth of undesired cell populations non-specifically which can then compete with the production of the required cell types or influence their function.

A relatively unexplored strategy to improve the efficiency of stem cell culture is to affinity target critical bioactive factors sequestered in biodegradable micro or nanoparticles to cell types of interest, thereby achieving a spatially and temporally controlled local "paracrine" stimulation of cells. Non-targeted biodegradable nano- and microspheres containing TGF-β1, TNF-α, and retinoic acid have been embedded with mouse embryonic stem cells (mESCs) grown in suspension as embryoid bodies to drive differentiation *in vitro* [7]. They have also been used to facilitate non-viral gene transfer to hESCs [8]. *In vivo*, paracrine signalling is pivitol to controlling cellular behaviour in a stem cell niche [9]. Niches are also likely to exist *in vitro*, such as has been proposed to occur in the context of bFGF signalling between differentiating and pluripotent hESCs mediating self-renewal [10,11]. Controlled release of bioactive factors can be achieved *in vivo* and *in vitro* using a spectrum of inorganic, or organic liposome- or polymer-based systems [12,13]. We have previously demonstrated targeted delivery of biodegradable encapsulates by functionalising nanoparticle surfaces with biotin:streptavidin-anchored epitope-specific antibodies and

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shown the prospective utility of this approach for T-cell stimulation, drug delivery, vaccination and immunotherapy [14–20], including targeted delivery of the Leukocyte Inhibitory Factor (LIF) for modulation of immune responses [17,21].

In the present study we evaluated the efficacy of affinity targeted biodegradable nanoparticles-mediated delivery of LIF, the essential factor for mESC self-renewal maintenance and pluripotency [22], as an alternative to its conventional daily replenishment in culture medium. Using two different biodegradable nanosystems that release LIF in a slow-sustained fashion—an established solid Poly(lactide-co-glycolic acid) (PLGA) polyester [17,21] and a recently described hydrogel-based liposomal system we term Nanolipogel (NLG) - [23,24] we demonstrate the feasibility of this approach for long-term support of embryonic stem cell renewal, an achievement which has significant implications for improving the efficacy of stem cell culture systems for research and industrial and clinical applications.

2. Materials and methods

2.1. mESC culture

HM1 mESC from the 129 mouse strain were cultured on plastic tissue culture well plates (Corning Inc.) coated with 0.1% gelatin (Sigma) in GMEM (Sigma) supplemented with 5% foetal calf serum (Sigma), 1% L-Glutamine (Invitrogen), 1% Non-Essential Amino Acids (Invitrogen), 1% Sodium Pyruvate (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma) at 37°C, 5% CO_2. For standard treatment medium was supplemented with soluble LIF (25 ng/ml; Millipore). Cells were passaged using 0.025% trypsin/EDTA for 3 min at 37°C.

2.2. Preparation of LIF-PLGA

Murine LIF was encapsulated in avidin-coated PLGA nanoparticles using a modified version of a previously described water/oil/water double emulsion technique [25]. Briefly, 50 μg LIF was dissolved in 200 μl PBS and added dropwise with vortexing to 100 mg PLGA in 2 ml dichloromethane. The resulting emulsion was added to 4 ml of aqueous surfactant solution containing 2.5 mg/ml PVA and 2.5 mg/ml avidin-palmitate bioconjugate [25], and sonicated to create an emulsion containing nano-sized droplets of polymer/solvent, LIF and surfactant. Solvent was removed by magnetic stirring at room temperature; hardened nanoparticles were then washed 3× in distilled water and lyophilized for long-term storage.

2.3. Preparation of NLG-LIF

The preparation of this system has been previously described [24]. Lyophilized liposomes were reconstituted with a solution containing 5% (w/v) polymer PEG-PLA-acrylate, 2.5 mg/mL Ciba Irgacure 2959 and 10 μg/ml LIF. The liposomes were then irradiated under UV light for 8 min with a Blak-Ray long wave ultraviolet lamp (Model B 100) at a 10 cm working distance. The resulting NLG were pelleted by centrifugation (5 min at 7200 rcf) and resuspended in PBS. Loaded lipogels were then conjugated by NHS/EDC chemistry with avidin; the centrifugation/resuspension procedure was repeated three times.

2.4. Affinity targeting nanoparticles

SSEA-1 targeted nanoparticles were formed by reacting avidin-coated nanoparticles in GMEM with 4 μl of biotin-anti-SSEA-1 (0.5 mg/mL; BioLegend) per mg of nanoparticles for 15 min and used immediately.

2.5. Nanoparticle characterisation

Nanoparticle size and morphology were analysed via scanning electron microscopy (SEM) and a Nanosight LM-10 imaging station. Release of LIF was measured by incubating nanoparticles in PBS at 37°C and measuring LIF concentrations in supernatant fractions by ELISA. Total encapsulation was approximated as the amount of LIF released over a seven-day period and percent encapsulation efficiency calculated as total encapsulation divided by maximum theoretical encapsulation.

2.6. Affinity targeted delivery to mESCs on mitotically inactivated feeder cells

mESCs (2.5×10^4 cells/cm^2) were plated onto wells of 4 chamber slides with MEFs in 500 μl of medium. After 24 h medium was replaced and cells were treated overnight with medium supplemented with 10 μg/ml SSEA-1 targeted (or untargeted, i.e. antibody-free) rhodo/biotin-avidin-adaptor NLG or PLGA. MEFs were

employed as negative control to evaluate whether the functionalised membrane provides a non-specific affinity targeting system. Rhodamine incorporation was assessed by live cell immunofluorescence.

2.7. Optimisation of affinity targeted delivery of LIF for mESC renewal in feeder-free culture

mESCs (2.5×10^4 cells/cm^2) were plated in gelatin-coated wells of 4 chamber slides. After 24 h cells were incubated overnight with SSEA-1 targeted LIF-loaded nanoparticles at nominal concentrations (1, 10, 100, 1000 μg/ml). In parallel, different concentrations of soluble LIF (5, 10, 25, 50 ng/ml) were used. 25 ng/ml of soluble LIF was set as positive control. No soluble LIF and empty nanoparticles (1000 μg/ml) were employed as negative controls. At the end of the incubation, nanoparticle-treated cells were washed twice in PBS and medium with no soluble LIF was replaced everyday for the following 5 days. Soluble LIF-supplemented medium was also replaced everyday over the experiment period. The effect of each treatment on cell pluripotent specific markers and proliferation were evaluated by immunocytochemistry for Oct-4 and WST-1 test (Calbiochem® Rapid Cell Proliferation Kit, Merck Biosciences).

2.8. Clonogenicity assessment

mESCs were plated at low density (500 cells/10 cm^2) onto gelatin-coated Petri dish. After 24 h cells were incubated overnight with SSEA-1 targeted LIF-loaded NLG or PLGA at the optimised concentration (10 μg/ml) prior or after trypsinisation. In parallel, cells were grown in absence or in presence of soluble LIF (25 ng/ml) to be used as negative and positive control. At the end of the incubation, nanoparticle treated cells were washed twice in PBS and medium with no soluble LIF was replaced everyday for 5 days. Soluble LIF-supplemented medium was also replaced everyday over the experiment period. Cells were fixed and stained by using the Alkaline Phosphatase detection kit (Millipore) and following the manifacturer's instructions.

2.9. Exemplification of long-term renewal in feeder-free culture

The optimal treatment with LIF-loaded PLGA and NLG was applied over a 5 weeks period once per week, following trypsin/EDTA dissociation and replating. No LIF and soluble LIF (25 ng/ml) were used as negative and positive control, respectively. Every 7 days cells were replated at 2.5×10^4 cells/cm^2. At the end of the treatment, cells were assessed for pluripotency markers, karyology and differentiation potential.

2.10. Growth rate analysis

mESCs were seeded, treated with the optimised concentration of targeted nanoparticles (10 μg/ml for both PLGA and NLG) and counted everyday for 7 days using a haemocytometer. Positive and negative controls cell growth was also evaluated. Cell number versus time were plotted in order to calculate the specific growth rates during the exponential growth phase for each treatment. Triplicate counts were gathered at each passage.

2.11. Electric cell-substrate impedance sensing (ECIS) assay

Impedance characterisation of mESC behaviour was assessed with the ECIS system (ECIS Zθ, Applied Biophysics). Cells were grown on eight-well ECIS arrays (8W10+; Applied Biophysics) containing 40 250-μm gold microelectrodes per well. Two ECIS arrays were recorded in parallel, for a total of 16 wells per experiment. Measurements were performed directly in cell culture medium, allowing real-time monitoring. Both the ECIS arrays and the measurement station were kept in an incubator with high humidity at 37°C and 5% CO_2. Prior to cell seeding, electrode arrays were coated with 0.1% gelatin. Cells were plated in suspension at a density of 5×10^4 cells/cm^2 in each well. After treatment (10 μg/ml nanoparticles), the resistance of the cell–electrode interface for each of the 16 wells was measured everyday for 30 min. During the measurement, a non-invasive electric field of 1 μA was applied. Cells grown in absence of LIF ($n = 3$) and in presence of soluble LIF ($n = 3$) were used as negative and positive control, respectively, and compared to cell treated with PLGA ($n = 3$), NLG ($n = 3$) and empty nanoparticles ($n = 3$). Impedance of a cell-free electrode in media ($n = 1$) was also measured as a background signal.

2.12. Immunoblotting

mESCs were plated on gelatin-covered 6-well plates (10^6 cells/well). The next day, the cells were washed with PBS and cultured with GMEM medium free of serum and LIF containing 1% L-Glutamine, 1% Non-Essential Amino Acids, 1% Sodium Pyruvate, 0.1 mM 2-mercaptoethanol. Four hours later, the cells were stimulated with soluble murine LIF (25 ng/ml), LIF-loaded nanoparticles (PLGA or NLG) or empty nanoparticles for 10 min and 24 h. After the induction, the cells were harvested with lysis buffer (10% Glycerol (Sigma), 3% SDS (Sigma), 62.5 mM TrisHCl (Sigma) pH 6.8, 0.005% Bromophenol blue (Sigma), 3% β-mercaptoethanol) and

proteins were resolved on a 10% SDS-polyacrylamide gel and trasferred to a nitro-cellulose membrane (BioRad Laboratories). The blot was blocked with TBS-T (20 mM Tris–HCl, pH 7.6, 136 mM NaCl (Sigma), and 0.1% Tween-20 (Sigma)) containing 5% skimmed milk or 3% BSA and then incubated with the primary antibody for 1 h at room temperature. After washing with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1/2 h at room temperature. Signals were detected with Pierce ECL Western Blotting Substrate (Pierce, Thermo Fisher Scientific). The antibodies used were phospho STAT-3 (Tyrosine 705; 1:1000; Cell Signalling), STAT-3 (1:1000; Cell Signalling) and Oct-4 (1:1000; Santa Cruz Biotechnology Inc.). Secondary antibodies were rabbit IgG and mouse IgG1 (1:5000; Amersham Biosciences). To quantify the level of activation of the protein in relation to the total protein loaded the software ImageJ was used on the developed X-ray films of the blots.

2.13. Immunostaining

Briefly, mESCs on were washed once with PBS and fixed with 4% PFA. After permeabilisation in 0.2% Igepal (Sigma) and blocking in 10% normal rabbit serum (Millipore), cells were incubated with primary antibodies Oct-4 (1:50; Santa Cruz Biotechnology) and Nanog (1:30; R&D systems) overnight at 4 °C. Visualisation with secondary antibodies was performed using Alexafluor antibodies 488 and 555 (Invitrogen), and nuclei were counterstained with 4′,6-diamidino-2-phenylindole, dilactate (DAPI). To stain live cells mESCs were washed once with GMEM medium supplemented with 0.1% BSA. Cells were then incubated with the directly conjugated antibody (SSEA-1 Alexafluor-488; 1:30, BioLegend) for 30 min at room temperature in the dark. Nuclei were counterstained with Hoechst. Imaging analysis was carried out using a Leica SPE microscope.

2.14. Flow cytometry analysis

Single cell preparation was obtained by incubation with 0.025% trypsin/EDTA for 5–10 min at 37°C, resuspended in FACS PBS (0.1% BSA, 0.1% sodium Azide in PBS) and incubated with directly conjugated antibody (SSEA-1 Alexafluor-488) for 20 min. Flow cytometry data were acquired using a Becton Dickinson FACSCalibur cytometer. The percentage of positive cells was established using FlowJo software by comparing experimental cells to control groups (no LIF- and standard condition-treated cells) at each passage.

2.15. RT-PCR

mESC RNA was prepared from snap–frozen cell pellets using Trizol® reagent (Invitrogen) and then samples were treated with RQ1 RNase-Free DNase (Promega). RNA concentration and purity were measured using NanoDrop Spectrophotometer (NanoDrop® ND1000). Complementary DNA was synthesized from 500 ng of total RNA using Superscript III reverse-transcription kit (Invitrogen) with oligo-dT primers. Mouse *Oct-4, Nanog, PEM* and *Sox-2* expression was determined using in 25 final volume using Taq DNA polymerase (Bioline Reagents Ltd.). β-actin was employed as reference gene. Primers used and their characteristics are listed in Table 1.

2.16. Undirected embryoid body (EB) mediated differentiation

Differentiation potential was assessed by undirected *in vitro* assay. mESCs (1000 cells per microwell) were aggregated in AggreWell plates (Stem Cell Technology Inc.) by centrifugation in serum-free conditions. One day later EBs were transferred into six-well Ultra-low Adherent plates (Stem Cell Technology) and grown in suspension for 7 days at 37 °C in 5% CO$_2$ in standard mES medium without LIF. Cells were then plated into 4well-culture slides (Becton Dickinson Labware) then grown at 37°C in 5% CO$_2$ in the same medium for one week.

Table 1
Oligonucleotide sequences used for RT-PCR analysis.

Markers	Accession number	Prod. Size (bp)	Sequences (5′ → 3′)	Annealing temperature
Oct-4	NM_013633	521	S:AAGGTGGAACCAACTCCCGA A:GTAGCCTCATACTCTTCTCG	62 °C
Nanog	NM_028016	620	S:AGCACCAGTGGAGTATCCCAG A:TCTTCCGAAGGTCAGGAGCTC	55 °C
PEM	NM_008818	514	S:TCCAGCCGCAAGGTCACCAGG A:TCCTCCTGGTTCTTCTGGCC	58 °C
Sox-2	NM_011443	362	S:CCGCGTCAAGAGGCCCATG A:AGCTGTCCATGCGCTGGTTC	56 °C
β-*actin*	NM_007393.3	242	S:CGTGGGCCGCCCTAGGCACCA A:TTGGCCTTAGGGTTCAGGGGGG	62 °C

Bp: base pairs; S: sense; A: antisense.

2.17. Teratoma induction

mESCs treated with PLGA and NLG for 5 passages were suspended at 5×10^6 cells per 100 μl in standard HEPES-buffered DMEM. Approximately 20 μl of this cell slurry was transferred under the kidney capsule of 129/Ola strain mice using a fine glass capillary. One month after injection, teratomas were dissected, fixed overnight in 10% phosphate-buffered formalin and embedded in paraffin. Sections were stained with Masson's Tricolour stain. Mice were housed in specific pathogen-free conditions and the care of the animals was in accordance with UK Home Office regulations.

2.18. Karyology

Cells, were plated 3 days prior to performing chromosome spreads and processed with the Karyomax kit (Invitrogen). Five different metaphase spreads were examined per treatment.

2.19. Statistical analysis

All statistical analyses were performed using the Statistics Package for the Social Sciences, version 11.0 (SPSS Inc., Chicago, IL, USA). The Kolmogorov−Smirnov test was used to determine whether the data were random samples from a normal distribution. For normally distributed variables, an unpaired *t*-test was applied. Replicates ($n = 3$ for each experiment, $n = 5$ for clonogenicity) were performed and the results represent mean ± SD. For ECIS measurements ANOVA followed by Tukey's multicomparison analysis was performed. A level of $P \leq 0.05$ was accepted as statistically significant and $P \leq 0.01$ as highly statistically significant. All data from ELISA are presented as the mean ± SD from triplicate samples at the specified time points.

3. Results

3.1. Characterisation of nanoparticle kinetics and targeting

PLGA and NLG nanoparticles containing LIF were functionalised for affinity targeted delivery of LIF to mESC by streptavidin-biotin cross-linkage of an antibody to SSEA-1 to particle surfaces (Fig. 1a, b). Nanoparticle preparations consisted of approximately 10^7 nanoparticles per mg of encapsulating matrix with an average diameter of 200 ± 50 nm. For both formulations the concentration of LIF was approximately 1 ng/mg equating to 10^{-7} ng or 2400 molecules of LIF per nanoparticle (LIF molecular weight = 25 kD). To insure that cells received the same dose of LIF regardless of formulation, the NLG polymer to lipid ratio was adjusted to yield similar kinetics as PLGA consisting of constant release over 4 days (shown for PLGA, Fig. 1 d) [17,24].

To assess the capacity of anti-SSEA-1 surface functionalised nanoparticles to achieve an affinity targeted delivery of an encapsulant to mESCs, we first delivered a pH sensitive dye that only fluoresces upon internalization (pH Rhodo, Invitrogen). mESCs grown on mouse embryonic fibroblasts (MEF), or MEF alone were exposed to nanoparticles at different concentrations (1, 10, 100 μg/ml) for 30 min, 1 h, 3 h and overnight. As a negative control cells were treated with untargeted nanoparticles (ie. no SSEA-1 antibody on their surface) for the same length of time. After 3 h or more of exposure to targeted and untargeted nanoparticles of both formulations at $\geq$100 μg/ml, non-specific rhodamine signal in SSEA-1-negative MEFs was observed. However, shorter exposures ($\leq$1 h) at lower concentrations (ie. 1 μg/ml) diminished rhodamine staining in SSEA-1 positive mESCs. These experiments suggested that affinity targeted stimulation of mixed cell populations requires optimisation. In this instance, the optimum conditions for affinity targeting of SSEA-1 positive mESCs on MEF for both nanoparticle formulations was overnight treatment at 10 μg/ml (Fig. 2a).

3.2. Short-term paracrine stimulation for mESC renewal in feeder-free culture

Our next objective was to demonstrate whether nanoparticles-mediated delivery of LIF could be beneficial in the short-term
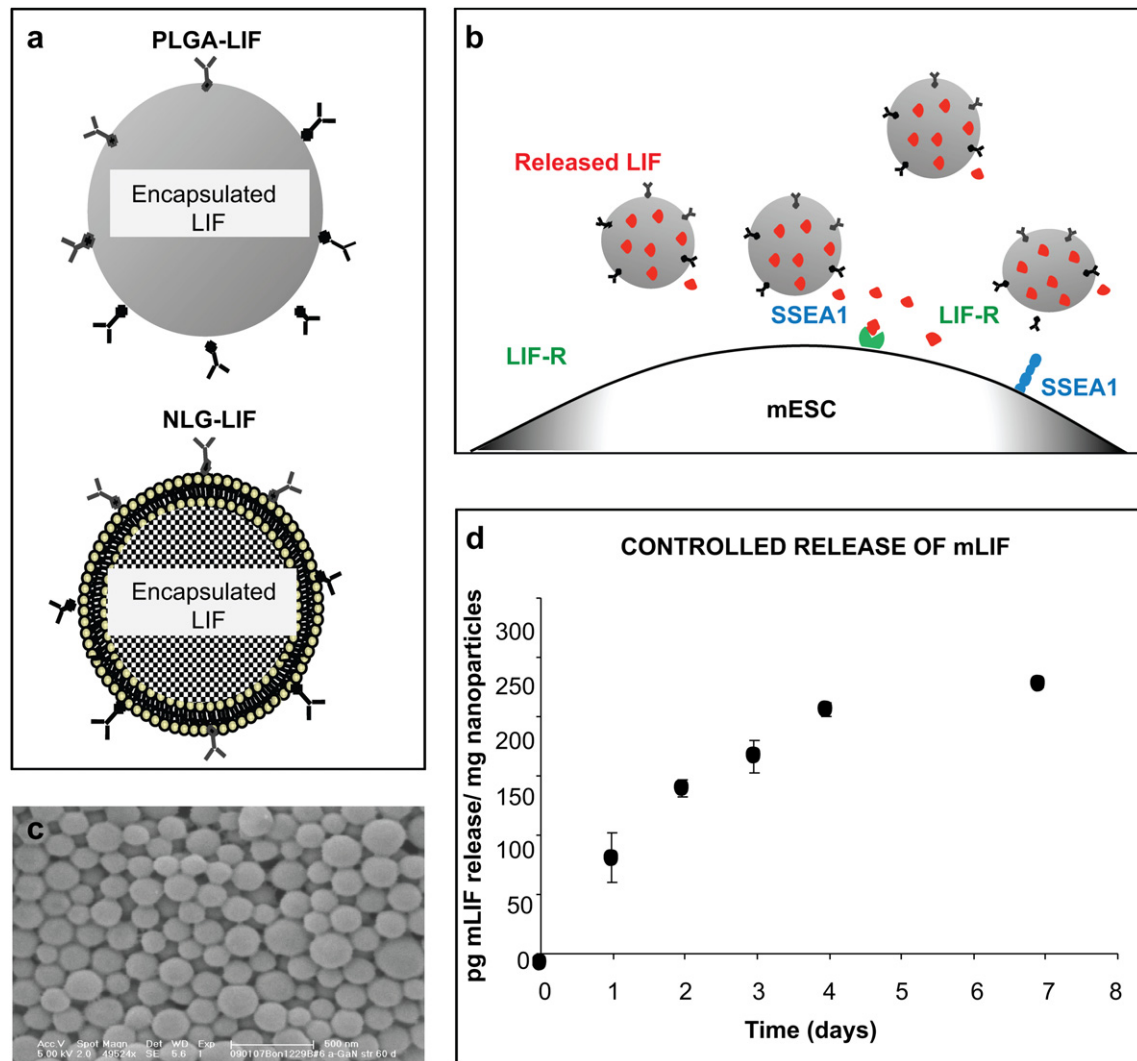
Fig. 1. Nanoparticle characteristics. (a) For both PLGA and NLG-LIF is encapsulated in a core, which is surrounded by a phospholipid bilayer shell for the latter. Surface anchored antibodies permit nanoparticle targeting. (b) Schematic of targeted activation of LIF receptor (LIF-R, green) on mESCs. Antibodies to SSEA-1 (black) on nanoparticles recognise SSEA-1 (blue). LIF released (red) during nanoparticle degradation activates LIF-R. (c) SEM of PLGA nanoparticles. (d) Cumulative controlled release of LIF from PLGA. Mean $\pm$ SD ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

context of mESC clonal outgrowth. mESCs were plated on gelatin-coated at low density following Trypsin/EDTA-mediated dissociation to single cells. Cells were treated with 10 μg/ml of SSEA-1 targeted nanoparticles overnight either pre- or post-passaging and then cultured for an additional 5 days with daily exchange of basal medium with no additional LIF added. Giemsa and Alkaline Phosphatase (ALP) staining was used to assess total cell colony frequency and the proportion which remained undifferentiated, respectively. Compared to the positive control wherein LIF was solublised conventionally, total colony frequency was significantly reduced in the negative control (no LIF) and all treatment groups ($P < 0.01$) with the exception of post-passage treatment with NLG-LIF. LIF delivered using the NLG formulation of nanoparticles also yielded as high a proportion of ALP+ colonies as the positive control, irrespective of whether cells were treated before or after passaging (Fig. 2 b). By contrast, pre- and post-passaging treatment with PLGA-LIF was comparable to LIF-negative control suggesting that in the context of low density outgrowth it was inferior to NLG.

We next considered nanoparticles-mediated delivery of LIF for purposes of mESC self-renewal under routine feeder-free conditions for cell expansion. For this, we adopted a protocol of treating cells

with nanoparticles overnight (~ 16 h) the first day after plating at a density of 2.5×10^4 cells/cm^2 followed by daily exchange of only the basal medium as for the preceding experiment (Fig. 2c). Treatments with SSEA-1 targeted NLG-LIF and PLGA-LIF nanoparticles at 10 μg/ml were compared with soluble LIF at 25 ng/ml, SSEA-1 targeted empty (LIF-free) nanoparticles at 1000 μg/ml and no LIF. After 5 days cultures were evaluated by immunostaining for pluripotency marker (Oct-4) and bioreduction of the stable tetrazolium salt, WST-1, the latter an indication of the number of metabolically active cells in culture (Fig. 3 a,b). In the absence of LIF (ie. no LIF supplementation of medium or treatment with of LIF-free SSEA-1 targeted nanoparticles, "Empty NP"), there was an anticipated qualitative reduction in the size and frequency of Oct-4-positive colonies concurrent with quantitative reduction in metabolic activity compared with soluble LIF supplementation ($P < 0.01$). By contrast treatment with PLGA and NLG-LIF supported pluripotent marker (Oct-4)-positive colony growth and metabolic activity, which was equivalent to soluble LIF (shown for NLG). To evaluate whether treatment with nanoparticles altered attachment to and coverage of the gelatin substrate provided, cultures were evaluated using Electric Cell-substrate Impedance Sensing (ECIS) [26,27]. By this method impedance of a non-invasive
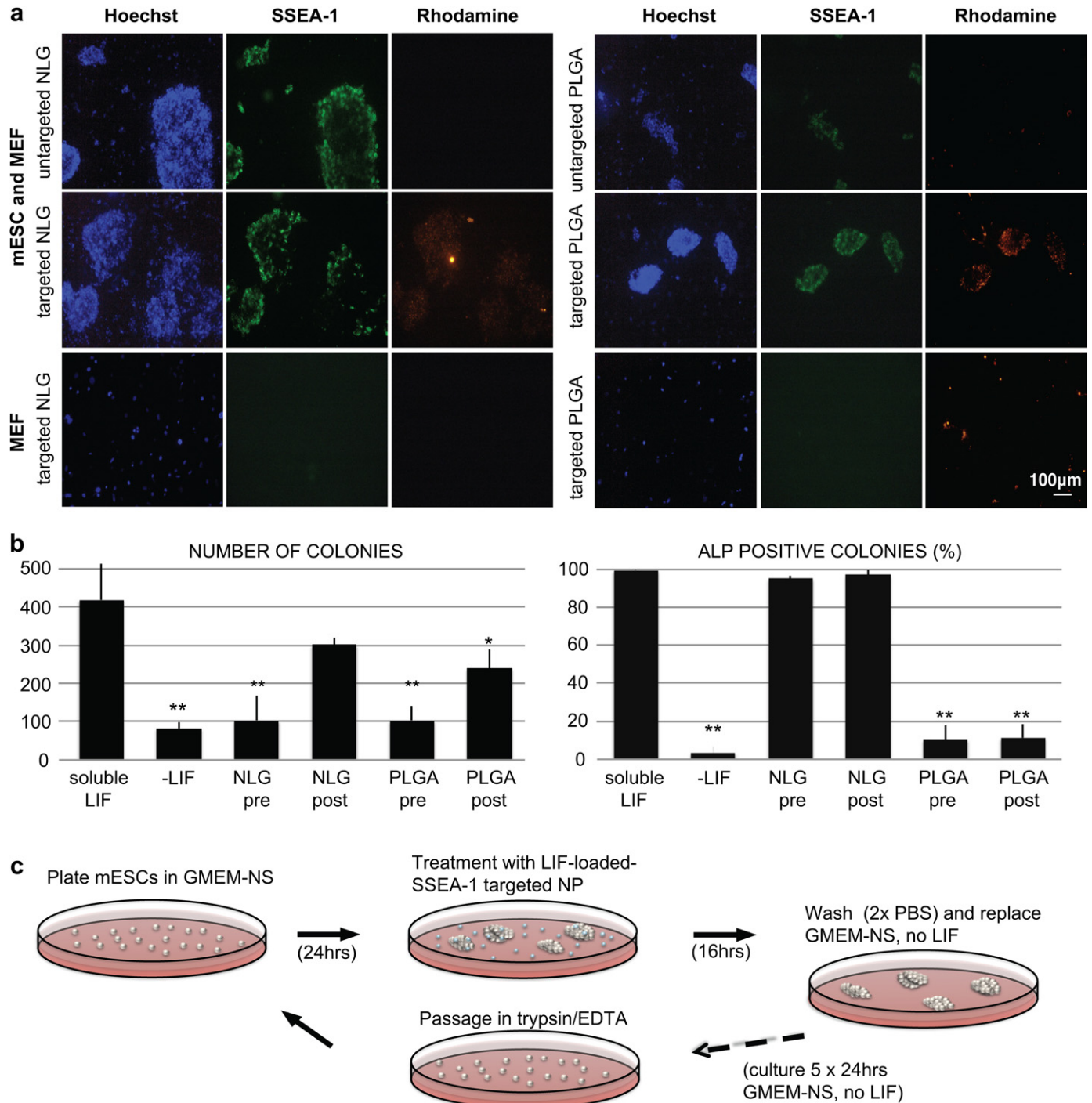
Fig. 2. Optimisation of affinity targeted LIF delivery. (a) Exposure of mESCs on MEF feeders or MEF alone to rhodamine-labelled nanoparticles (10 μg/ml). Cells were incubated with rhodamine-NLG or PLGA without (untargeted) or with (targeted) coating of anti-SSEA-1 antibody for affinity targeting to mESCs. A red channel signal resulting from endocytosis of nanoparticles is only observed in SSEA-1+ (green) colonies after treatment with targeted nanoparticles. (b) LIF provision by SSEA-1 targeted nanoparticles pre- and post-plating of mESCs reveals treatment and formulation dependent support of total and ALP+ colony outgrowth (left and right, respectively). Mean values ± SD ($n = 5$) normalised to soluble LIF (25 ng/ml). *$P \leq 0.05$, **$P \leq 0.01$. (c) Optimised feeder-free culture regime for mESCs cultured in basic medium with affinity targeted nanoparticle mediated delivery of LIF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

electrical current applied to cells grown on a substrate coated microelectrode serves as a measure of cell coverage and integrity. ECIS revealed no significant differences ($P > 0.05$) between targeted LIF-nanoparticle treated cultures and the soluble LIF positive control after 3 days of growth. In contrast, cell impedance was significantly lower in cells treated with targeted empty nanoparticles and no soluble LIF-negative controls ($P < 0.01$) (Fig. 4 a).

To confirm the bioactivity of nanoparticle mediated delivery of LIF we evaluated the phosphorylation of STAT-3 by Western blotting. Cells stimulated with soluble LIF-supplemented medium resulted in a pronounced 300-fold increase in STAT-3 phosphorylation within 10 min, as previously reported [28]. Treatment with either targeted nanoparticle formulation was comparable to negative controls. However, after 24 h, STAT-3 phosphorylation in
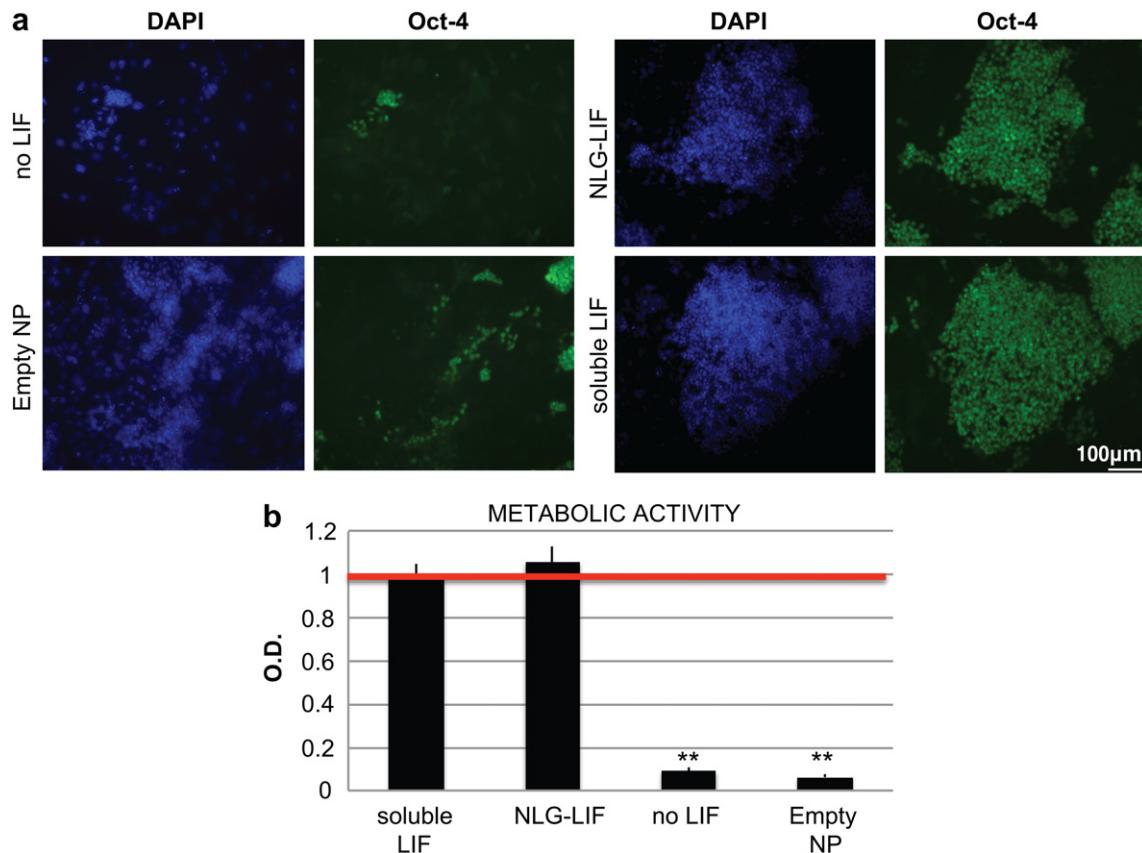
Fig. 3. Effect of LIF nanoparticles on mESC viability. (a) NLG-LIF (10 μg/ml) support of expression of Oct-4 after 5 days of culture in a manner similar to daily supplementation of basal medium with soluble LIF (25 ng/ml). Negative controls (no LIF) or LIF-free NLG (empty NP) are also shown. (b) WST-1 test indicating relative levels of metabolic activity for mESCs treated with soluble LIF (25 ng/ml), NLG-LIF (10 μg/ml), no LIF and LIF-free NLG (empty NP). Signal (optical density: O.D.) normalised to positive control; error bars, SD ($n = 3$), **$P \leq 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NLG-LIF treated cells was at least two-fold higher than in the absence of LIF whereas PLGA-LIF and LIF-supplemented medium before replenishment were comparable to the absence of LIF (Fig. 4b). Consistent with these experiments, the growth kinetics of the NLG-LIF over the first 6 days of culture more closely mimicked that observed for soluble LIF, whereas growth of PLGA supplied LIF appeared to lag. By 7 days however, both nanoparticle formulations supported more cumulative growth than in the absence of LIF (Fig. 4c).

3.3. Long-term nanoparticles-mediated delivery of self-renewal factors to stem cells supports pluripotency

Despite the apparent superior properties of the NLG formulation to support mESC clonal outgrowth, STAT-3 phosphorylation and feeder-free growth over short-term culture, we evaluated both formulations using the optimised treatment protocol (Fig. 2c) for competence to provide longer-term support over successive passaging. After 5 weeks of continuous culture, in the course of which cells were passaged 5 times and treated with NLG-LIF or PLGA-LIF overnight once after each passage, maintenance of an undifferentiated cell phenotype was confirmed by immunocyto-chemistry for Oct-4, and Nanog, RT-PCR for these markers, *PEM* and *Sox-2*, and flow cytometry for SSEA-1. By contrast, mESCs cultured in the absence of LIF over this period manifest qualitative changes consistent with loss of an undifferentiated phenotype including reduction in colony size and pluripotency marker gene expression (Fig. 5 and Fig. 6, a–c). For both formulations, karyology analysis of cells also confirmed a normal karyotype of 40 XY chromosomes in

all of 30 spreads for each LIF-treatment group. Following embryoid body mediated differentiation *in vitro*, cells also retained competence to forming beating cardiomycyte-like cells (shown for NLG-LIF, online Video 1). Finally, analysis of differentiation potential at this stage by injection of PLGA- and NLG-LIF treated mESCs under the kidney capsule of the same strain of mice (129/Ola) confirmed their potential to generate teratomas composed of derivatives of all three germinal lineages (Figs. 5 and 6, d).

Supplementary video related to this article can be found online at doi:10.1016/j.biomaterials.2012.06.011.

4. Discussion

Our study reports an alternative approach to stem cell culture focused on the use of biodegradable nanoparticles for affinity targeted paracrine stimulation. This approach offers several advantages to conventional solubilisation of bioactive factors in media. First, a high density of agent can often be encapsulated, dispersed or dissolved within nanoparticles which, depending on the preparation process, can be engineered to yield different properties and release characteristics for the entrapped agent [29–33]. Second, because of the versatility of chemistries and preparation methods in these systems, surface functionalities can be incorporated into the nanoparticle, facilitating additional attractive properties such as attachment of target ligands for interaction with specific cells or tissues. Such targeted delivery protects the agent from the surrounding medium, focuses the dose and delivers it in a paracrine fashion in the case of extracellular targeting, or enhances the intracellular dose upon endocytosis in internalizing cells [17,34].
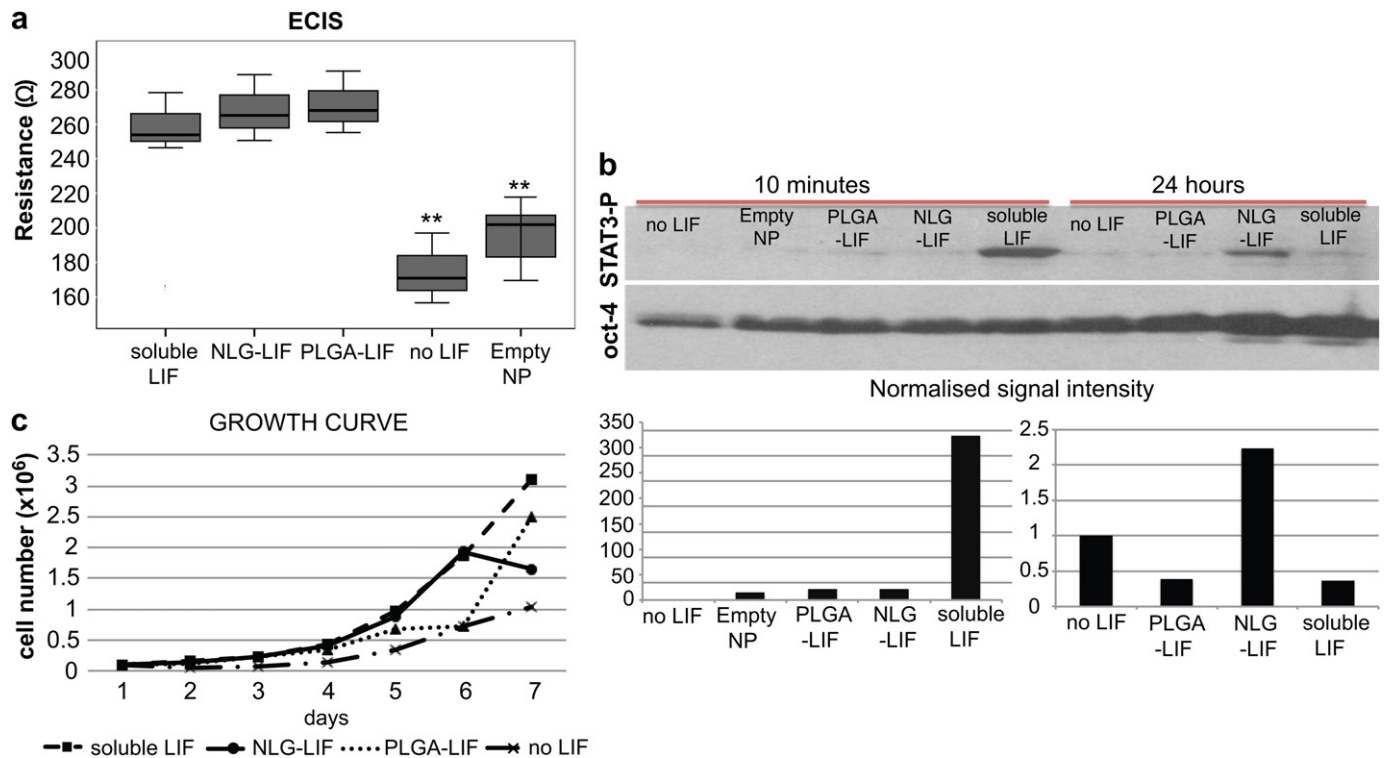
Fig. 4. Evaluation of mESC attachment and LIF-nanoparticle mediated signalling and growth kinetics. (a) ECIS assay of mESCs cultured in medium supplemented with soluble LIF, or treated with PLGA- or NLG-LIF, as compared against no soluble LIF (no LIF) or LIF-free nanoparticles (empty NP). High resistance indicates good cell growth and hence electrode coverage. **$P \leq 0.01$. (b) STAT-3 phosphorylation. Upper panel: Western blotting of phosphorylated STAT-3 in mESCs 10 min and 24 h after treatment with medium lacking (no LIF) or containing soluble LIF, or with empty (empty NP) or PLGA- or NLG-LIF. Middle panel shows levels of Oct-4 for comparison. Lower panel: Quantification of STAT-3 phosphorylation data normalised to no LIF at 10 min or 24 h post-treatment. (c) Supplementation of medium with NLG- or PLGA-LIF, or soluble LIF promotes cell growth over unsupplemented medium (no LIF) for a period of 7 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

One of the most striking ramifications arising from the use of affinity targeted nanoparticles for the sustained release of bioactive factors to achieve paracrine stimulation is the opportunity it provides to reduce the quantity of bioactive factors consumed in culture. In the case of our experiments, we calculated that conventional culture of mESCs consumes 25 ng of LIF/ml/day, amounting to 875 ng after 5 weeks of culture maintaining only a single ml of cultured cells throughout. In contrast, using an optimised concentration of 10 μg of NLG-LIF or of PLGA-LIF per week, containing 0.01 ng of LIF, only 0.05 ng of LIF would be consumed to achieve the same level of growth, a striking 1.74×10^4-fold reduction. To our knowledge this level of reduction in the utilisation of a critical bioactive factor such as LIF in long-term culture with successive passaging has not been achieved by other methods of culture, such as for example by immobilisation on biocarriers such as thin planar films of maleic anhydride copolymer or viral polyhedron protein surfaces [35,36]. Compared with delivery by biodegradable encapsulant immobilised ligands are exposed and thus more likely to degrade in culture media. Immobilisation also precludes interaction with cells that are not in direct contact. This would be a natural consequence of successive division of cells growing as a domed colony as for mESC. This would result in differentiation and preclude long-term support of self-renewal, although short-term culture is still tenable [35,36]. As bioactive factors such as growth factors and cytokines are probably one of the most expensive components of cell culture media the means to control their delivery, bioactivity and usage would be of significant benefit to translational industrial and clinical applications requiring extensive culture of stem cells or derivatives, especially where there is a reliance on complex cocktails of factors.

Our experience suggests that in complex culture systems, for example in feeder-dependent systems, applying affinity targeted stimulation may require optimisation of treatment dosage. Theoretically, the temporal and spatial control this culture system offers for the provision of bioactive factors could also be applied in other contexts such as the positive or negative selection of cell subpopulations in a heterogeneous culture environment, the expansion of rare cells from primary tissue isolates and directed differentiation. It is likely that placing an emphasis on targeted paracrine stimulation could also alter the apparent requirement for bioactive factors established by conventional culture systems. This could generate an intellectual property pertaining to any given process.

To establish the generality of this paracrine delivery approach we attempted to evaluate two types of nanoparticle formulations as biodegradable vectors so as demonstrate that the benefits of paracrine stimulation was not necessarily a materials based phenomenon. For this, we used well-established polyesters, exemplified by PLGA, which are commonly used as biodegradable vectors and are known for their safety of use in humans. Their degradation rate and the corresponding entrapped agent release rate vary from days (PGA) to months (PLA) and are easily manipulated by varying the ratio of PLA to PGA. PLGA nanoparticles can also be formulated in a variety of ways that improve targeting to specific cells [17,25]. The second class of carriers that are commonly used in drug delivery applications are lipid-based. They are exemplified by liposomes or variants thereof such as the lipogel (liposome with a polymeric interior) used in this study, which are easily modified for encapsulation of small hydrophilic or hydrophobic molecules, and even nucleic acids and proteins [37,38]. The safety profiles of both platforms comply in principle with regulatory
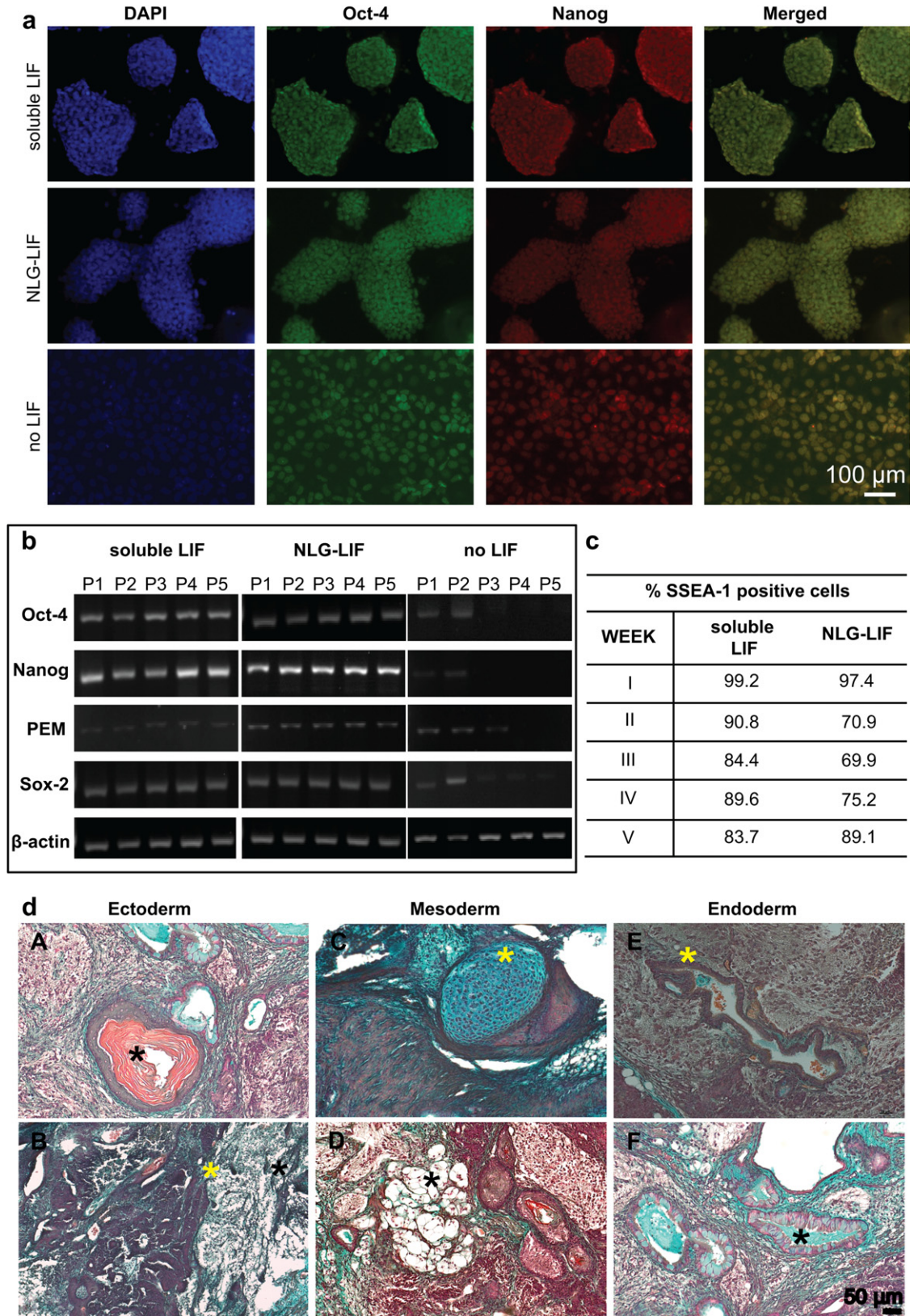
Fig. 5. NLG-mediated provision of LIF over 5 weeks' culture. (a) Nuclear DNA (DAPI) and immunocytochemical staining for Oct-4 and Nanog of mESCs that have been treated with soluble LIF, NLG-LIF or no LIF. Scale bar: 100 μm (b) RT-PCR. *Oct-4, Nanog, PEM* and *Sox-2* expression in mESCs treated with NLG-LIF vs soluble or no LIF. *β-actin* used as reference gene. (c) Flow cytometry indicates variable but high maintenance of SSEA-1 in mESCs treated with NLG-LIF, similar to mESCs conventionally maintained with soluble LIF. (d) Teratomas generated from mESCs treated with NLG-LIF for 5 weeks' culture prior to injection. Ectoderm: (A) keratinised epithelium and (B) immature neuroectoderm (yellow asterisk) and differentiated neural tissue (black asterisk). Mesoderm: (C) cartilage and (D) adipose tissue. Endoderm: (E) pulmonary epithelium and (F) gut epithelium. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
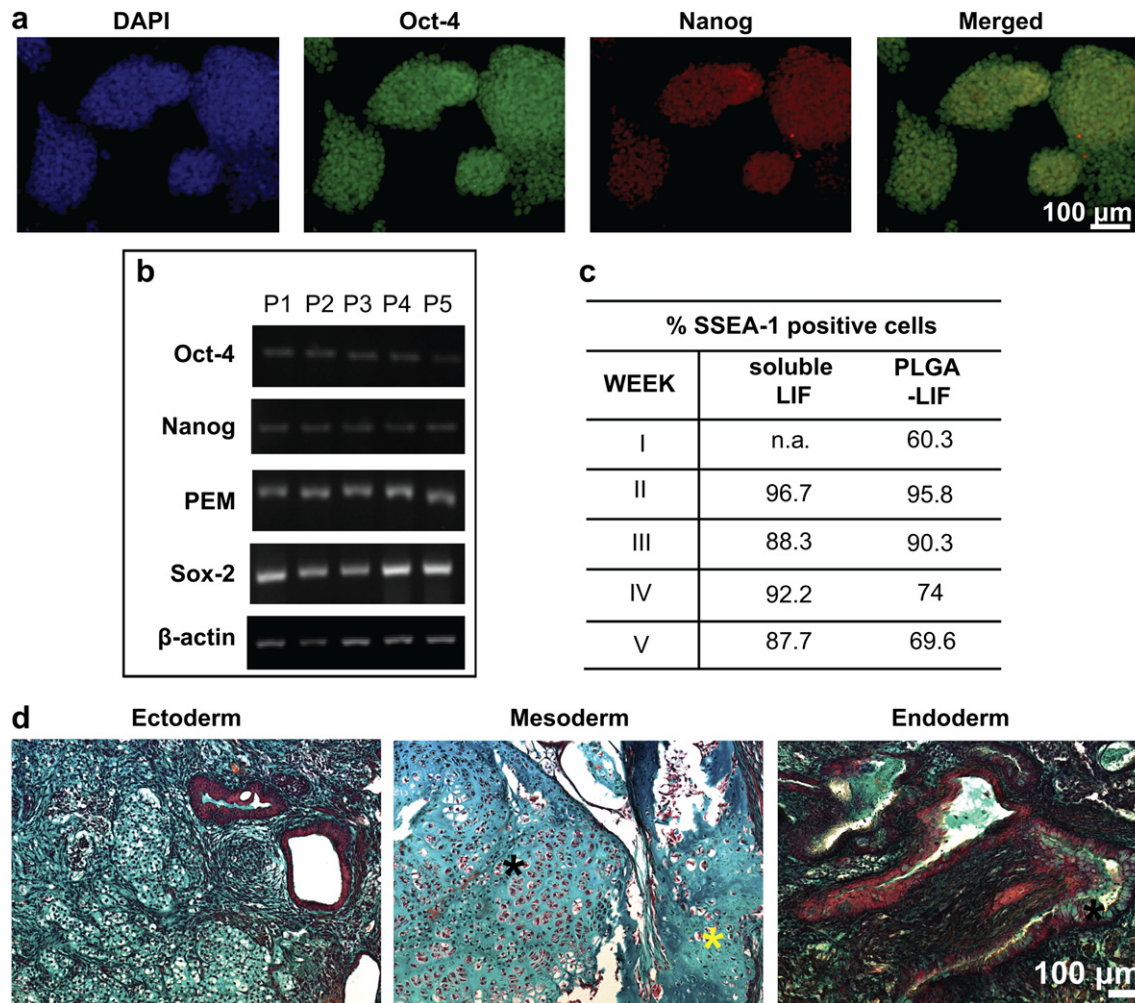
Fig. 6. PLGA-mediated provision of LIF over 5 weeks' culture. (a) Nuclear (DAPI) and immunocytochemical staining for Oct-4 and Nanog of mESCs that have been treated with PLGA-LIF nanoparticles. (b) RT-PCR. *Oct-4, Nanog, PEM* and *Sox-2* expression in mESCs treated with PLGA-LIF. *β-actin* was used as reference gene. (c) Flow cytometry indicates variable but high maintenance of SSEA-1 in mESCs treated with PLGA-LIF over 5 weeks, similar to mESCs conventionally maintained with soluble LIF. (d) Teratomas generated from mESCs treated with PLGA-LIF nanoparticles for 5 weeks' culture prior to injection. Mature neural tissue for ectoderm, bone (yellow asterisk) and cartilage (black asterisk) for mesoderm, gut epithelium for endoderm. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

requirements for safety in stem cell manufacturing for therapeutic applications. Our results from short-term culture experiments suggested that LIF bioactivity might have been better preserved by the NLG formulation as compared with PLGA. This may be due to the amount of glycolic acid and lactic acid by products of the polymer released during degradation. Compared with the PLGA system, the NLG system encapsulates LIF using 70% less polymer [24] by weight. A second possibility for the discrepancy in short-term function may be simply due to the nature of the nano-particles themselves. PLGA are solid biodegradable nanoparticles compared to the vesicular, fluid bilayer surface of the NLG. As such, the cell–nanoparticle interface in the short-term may be affected by the mechanics of this interaction. In long-term culture however, both particle formulations sustained mESC pluripotency, and demonstrated the feasibility of a paracrine stimulation focused approach to stem cell culture.

5. Conclusions

Our study illustrates an alternative paradigm for stem cell culture, featuring artificial temporal and spatially controlled para-crine stimulation with the potential to improve the efficacy of stem cell culture. This system is capable of significantly reducing the consumption of a single critical bioactive factor in the context of adherent cell culture in conventional tissue culture plasticware. We anticipate this approach is likely to be adaptable to other stem cell culture objectives, models and culture systems. As such we believe the nanoparticles-mediated delivery of growth factors in cell culture should contribute significantly to the scaleable manufacture of stem cells and the clinical delivery of new advanced cellular therapies for regenerative medicine.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2012.06.011.

References

[1] Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, et al. Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 2006;24(2):185—7.

[2] Yao S, Chen S, Clark J, Hao E, Beattie GM, Hayek A, et al. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proc Natl Acad Sci U S A 2006;103(18):6907—12.

[3] Suter DM, Krause KH. Neural commitment of embryonic stem cells: molecules, pathways and potential for cell therapy. J Pathol 2008;215(4):355—68.

[4] McIntosh K, Zvonic S, Garrett S, Mitchell JB, Floyd ZE, Hammill L, et al. The immunogenicity of human adipose-derived cells: temporal changes in vitro. Stem Cells 2006;24(5):1246—53.

[5] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284(5411):143—7.

[6] Tsutsui H, Valamehr B, Hindoyan A, Qiao R, Ding X, Guo S, et al. An optimized small molecule inhibitor cocktail supports long-term maintenance of human embryonic stem cells. Nat Commun 2011;2:167.

[7] Carpenedo RL, Seaman SA, McDevitt TC. Microsphere size effects on embryoid body incorporation and embryonic stem cell differentiation. J Biomed Mater Res A 2010;94(2):466—75.

[8] Green JJ, Zhou BY, Mitalipova MM, Beard C, Langer R, Jaenisch R, et al. Nanoparticles for gene transfer to human embryonic stem cell colonies. Nano Lett 2008;8(10):3126—30.

[9] Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: stem cells and their niche. Cells 2004;116(6):769—78.

[10] Bendall SC, Stewart MH, Menendez P, George D, Vijayaragavan K, Werbo-wetski-Ogilvie T, et al. IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. Nature 2007;448(7157): 1015—21.

[11] Peerani R, Rao BM, Bauwens C, Yin T, Wood GA, Nagy A, et al. Niche-mediated control of human embryonic stem cell self-renewal and differentiation. EMBO J 2007;26(22):4744—55.

[12] Fahmy TM, Fong PM, Goyal A, Saltzman WM. Targeted for drug delivery. Nano Today 2005;8:18—26.

[13] Yoo JW, Irvine DJ, Discher DE, Mitragotri S. Bio-inspired, bioengineered and biomimetic drug delivery carriers. Nat Rev Drug Discov 2011;10(7):521—35.

[14] Demento S, Steenblock ER, Fahmy TM. Biomimetic approaches to modulating the T cell immune response with nano- and micro- particles. Conf Proc IEEE Eng Med Biol Soc 2009;2009:1161—6.

[15] Demento SL, Bonafe N, Cui W, Kaech SM, Caplan MJ, Fikrig E, et al. TLR9-targeted biodegradable nanoparticles as immunization vectors protect against West Nile encephalitis. J Immunol 2010;185(5):2989—97.

[16] Park J, Fong PM, Lu J, Russell KS, Booth CJ, Saltzman WM, et al. PEGylated PLGA nanoparticles for the improved delivery of doxorubicin. Nanomedicine 2009; 5(4):410—8.

[17] Park J, Gao W, Whiston R, Strom TB, Metcalfe S, Fahmy TM. Modulation of CD4+ T lymphocyte lineage outcomes with targeted, nanoparticle-mediated cytokine delivery. Mol Pharm 2011;8(1):143—52.

[18] Park J, Mattessich T, Jay SM, Agawu A, Saltzman WM, Fahmy TM. Enhancement of surface ligand display on PLGA nanoparticles with amphiphilic ligand conjugates. J Control Release 2011;156(1):109—15.

[19] Steenblock ER, Fadel T, Labowsky M, Pober JS, Fahmy TM. An artificial antigen-presenting cell with paracrine delivery of IL-2 impacts the magnitude and direction of the T cell response. J Biol Chem 2011;286(40):34883—92.

[20] Steenblock ER, Fahmy TM. A comprehensive platform for ex vivo T-cell expansion based on biodegradable polymeric artificial antigen-presenting cells. Mol Ther 2008;16(4):765—72.

[21] Gao W, Thompson L, Zhou Q, Putheti P, Fahmy TM, Strom TB, et al. Treg versus Th17 lymphocyte lineages are cross-regulated by LIF versus IL-6. Cell Cycle 2009;8(9):1444—50.

[22] Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, et al. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 1988;336(6200):688—90.

[23] Metcalfe SM, Fahmy TM. Targeted nanotherapy for induction of therapeutic immune responses. Trends Mol Med 2012;18(2):72—80.

[24] Park J, Stern E, Wrzesinski SH, Flavell RA, Fahmy T. Nanolipogel combination delivery of small molecule inhibitors and cytokine enhances tumor immunotherapy. Nat Mater, in press.

[25] Fahmy TM, Samstein RM, Harness CC, Mark Saltzman W. Surface modification of biodegradable polyesters with fatty acid conjugates for improved drug targeting. Biomaterials 2005;26(28):5727—36.

[26] Bagnaninchi PO, Drummond N. Real-time label-free monitoring of adipose-derived stem cell differentiation with electric cell-substrate impedance sensing. Proc Natl Acad Sci U S A 2011;108(16):6462—7.

[27] Wegener J, Keese CR, Giaever I. Electric cell-substrate impedance sensing (ECIS) as a non invasive means to monitor the kinetics of cell spreading to artificial surfaces. Exp Cell Res 2000;259(1):158—66.

[28] Burdon T, Chambers I, Stracey C, Niwa H, Smith A. Signaling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells. Cells Tissues Organs 1999;165(3—4):131—43.

[29] Huang YC, Liu TJ. Mobilization of mesenchymal stem cells by stromal cell-derived factor-1 released from chitosan/tripolyphosphate/fucoidan nanoparticles. Acta Biomater 2012;8(3):1048—56.

[30] Kim SE, Jeon O, Lee JB, Bae MS, Chun HJ, Moon SH, et al. Enhancement of ectopic bone formation by bone morphogenetic protein-2 delivery using heparin-conjugated PLGA nanoparticles with transplantation of bone marrow-derived mesenchymal stem cells. J Biomed Sci 2008;15(6):771—7.

[31] Roger M, Clavreul A, Venier-Julienne MC, Passirani C, Sindji L, Schiller P, et al. Mesenchymal stem cells as cellular vehicles for delivery of nanoparticles to brain tumors. Biomaterials 2010;31(32):8393—401.

[32] Tatard VM, Menei P, Benoit JP, Montero-Menei CN. Combining polymeric devices and stem cells for the treatment of neurological disorders: a promising therapeutic approach. Curr Drug Targets 2005;6(1):81—96.

[33] Wu X, Zhang J, Yi D, Gu C, Wei X, Wu H, et al. Enhancement of proliferation and differentiation of bone mesenchymal stem cells by basic fibrous growth factor controlled release nanoparticles. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi 2010;27(1):80—5.

[34] Deb KD, Griffith M, Muinck ED, Rafat M. Nanotechnology in stem cells research: advances and applications. Front Biosci 2012;17:1747—60.

[35] Alberti K, Davey RE, Onishi K, George S, Salchert K, Seib FP, et al. Functional immobilization of signaling proteins enables control of stem cell fate. Nat Methods 2008;5(7):645—50.

[36] Nishishita N, Ijiri H, Takenaka C, Kobayashi K, Goto K, Kotani E, et al. The use of leukemia inhibitory factor immobilized on virus-derived polyhedra to support the proliferation of mouse embryonic and induced pluripotent stem cells. Biomaterials 2011;32(14):3555—63.

[37] Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol 2007;2(12): 751—60.

[38] Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 2005;4(2):145—60.