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Sustained Release of Recombinant Human Growth Hormone from Bioresorbable Poly(ester urea) Nanofibers

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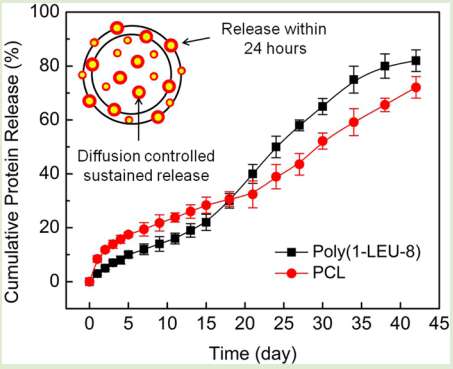
ABSTRACT: Recombinant human growth hormone (rhGH) has been used clinically for several decades. However, rhGH has a short half-life *in vivo* and therefore for efficacy requires frequent subcutaneous injection, leading to pain and poor patient compliance. An effective rhGH delivery system able to continuously release rhGH over a comparatively long period (generally over a month) would be a significant clinical advance. In this study, a novel long-duration release strategy of rhGH was developed by encapsulating sugar glass-stabilized rhGH in electrospun, bioresorbable poly(ester urea) (PEU) nanofibers. The rhGH was found to be randomly dispersed among the fibers, and sustained rhGH release with only a modest burst was observed for at least 6 weeks as studied by a bicinchoninic acid (BCA) protein assay. Significantly, the released rhGH remained bioavailable and bioactive as determined by an Nb2 cell bioassay specific for rhGH. Our results suggest the feasibility of this system as an effective long-term sustained release strategy for rhGH and potentially other therapeutic proteins.



Protein therapeutics possess advantages over small-molecule approaches including high target specificity and low off-target effects with normal biological processes.[1−4] As a protein drug, human growth hormone (hGH) has a unique role in promoting longitudinal bone growth and is widely used for the clinical treatment of short stature in children caused by growth hormone deficiency or growth failure.[5−13] However, the current rhGH standard of care requires frequent subcutaneous injection (i.e., three times a week or daily) as a consequence of its short half-life, which leads to poor patient compliance, higher than desired dose, and increased cost.[14−17] Therefore, a sustained-release rhGH formulation would not only provide improved patient compliance but also alleviate the costly burden associated with frequent injections. The best known rhGH-sustained delivery system, Nutropin Depot, which is encapsulation of rhGH in biodegradable poly(lactic acid-*co*-glycolic acid) (PLGA) microspheres, was approved by the US FDA in 1999 as a monthly drug.[18−20] Later in 2004, this product was withdrawn from the market due to several issues, including low loading efficiency, high burst release, protein denaturation during microsphere preparation, and inflammation from acidic PLGA degradation byproducts.[18,20] So far, it has remained a challenge to develop an effective rhGH delivery system that lasts longer than a month.

Electrospun nanofibers have attracted increasing attention in the controlled delivery of bioactive molecules, such as proteins,[21−24] growth factors,[23,25−27] and genes.[28−30] As delivery carriers, electrospun nanofibers offer many advantages including:[31,32] (1) high drug loading efficiency, (2) large pores to enhance drug diffusion to the surrounding medium, and (3) the potential for delivering various bioactive cocktails. Significantly, sustained release can be achieved by properly modulating the biodegradability or hydrophilicity of the polymers, as well as the diameters and porosities of the nanofibers.[33] Despite these advantages, problems associated with electropun nanofibers include the potential loss of protein bioactivity during electrospinning and the inflammatory reactions of the surrounding tissues caused by acidic degradation of the polymer nanofibers. Therefore, stabilizing the protein prior to electrospinning and selecting a polymer matrix that does not produce acidic degradation byproducts are necessary to make an effective sustained delivery system for protein drugs like rhGH.

Recently, a novel sugar glass nanoparticle (SGnP) system for stabilizing proteins in drug delivery systems was reported by Giri et al.[34] This sugar glass technology was shown to yield excellent stabilization of lysozyme from process-related stresses with little or no denaturation, high encapsulation efficiency, and burst-free sustained release. Poly(ester urea)s (PEUs) are resorbable amino acid based polymers that have shown tunable degradation with nonacidic byproducts.[35−40] We therefore hypothesized that the sugar glass nanoparticle stabilized rhGH

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encapsulated in the PEU nanofibers could serve as a new sustained release strategy for rhGH. In this work, sugar glass stabilized rhGH nanoparticles were fabricated and electrospun into poly(1-LEU-8) PEU nanofibers. Sustained release of rhGH from the PEU nanofibers was studied over a period of 6 weeks *in vitro*. The bioactivity of the released rhGH was determined by an Nb2 cell bioassay. The release and bioactivity of rhGH from nanofibers made of PCL, one of the mostly studied degradable polymers that have been used for several human clinical applications,[41] was also studied as a control.

SGnPs were prepared from an inverse micelle of dioctyl sulfosuccinate (AOT) according to methods described previously,[34] as demonstrated in Figure 1(a). Earlier works

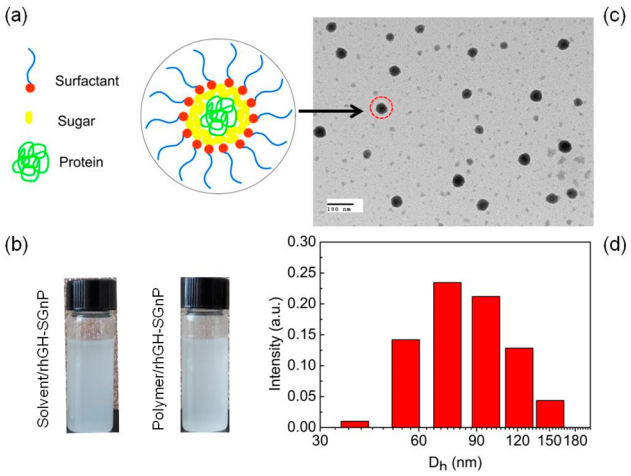

Figure 1. (a) Schematic presentation of a biomolecule encapsulated in a SGnP. (b) rhGH-SGnPs suspended in isooctane (left) or PEU polymer solution in 1,4-dioxane (right). (c) Representative TEM image of rhGH-SGnP. (d) Particle size distribution of rhGH-SGnP from DLS.

have reported that [water]/[surfactant] mole ratio (w = [H_2O]/[AOT]) plays a significant role in determining the equilibrium micelle size. The [water]/[surfactant] mole ratio can be varied from 10 to 15, and the mass ratio of protein to trehalose can be tuned from 1:500 to 1:200 to provide sufficient coating of sugar glass around the protein without adversely affecting performance. In this study, to maximize rhGH encapsulation while maintaining the stabilization effects, the [water]/[surfactant] mole ratio and mass ratio of protein to trehalose were 15:1 and 1:200, respectively. This combination of w and mass ratio of protein to trehalose resulted in particle size distributions of 64 ± 13 nm, as measured from TEM (Figure 1(c)), and 81 ± 20 nm as determined by DLS (Figure 1(d)). DLS typically reports larger values than TEM, which is due to the extended surfactant chains in the solution state. The freeze-dried nanoparticles can be reintroduced into organic solvents or polymer solutions as a stable suspension. Figure 1(b) showed that sugar glass stabilized rhGH nanoparticles can be homogeneously suspended in isooctane or a 12 wt % PEU polymer solution in 1,4-dioxane.

Poly(1-LEU-8) PEU and PCL nanofibers without protein and with protein and fluorescent dye loaded nanofibers were produced by electrospinning. The electrospinning parameters were optimized by conducting a series of systematic studies on the effects of flow rate, polymer/protein concentration, applied voltage, and jet stability on the size and morphology of the resulting polymer nanofibers. To fabricate nanofibers without protein encapsulation, the desired fiber morphologies can be obtained by electrospinning a 10 wt % PEU in HFIP or a 12 wt % PCL in DCM:DMF (4:1 v/v). However, to fabricate protein/dye nanoparticles encapsulated polymer nanofibers, the optimized parameter was found to be 7 wt % PEU polymer solution with 1.6 wt % protein/dye nanoparticles in 1,4-dioxane:EtOH = 2:1 (v/v) or 7 wt % PCL polymer solution with 1.6 wt % protein/dye nanoparticles in DCM:EtOH = 3:1 (v/v). A dioxane and EtOH mixture was used instead of HFIP or DMF for the protein loaded fiber electrospinning since the sugar glass nanoparticles could act as a barrier between the protein and organic solvent if the solvent polarity index is less than 5.[34] Dioxane, DCM, and EtOH have a polarity index of 4.8, 3.4, and 4.3, respectively. Thus, the solvent mixture of dioxane:EtOH = 2:1 (v/v) and DCM:EtOH = 3:1 (v/v) has a polarity index less than 5, which is favorable for protein bioactivity reservation during the electrospinning process.

The fiber size, morphology, and sugar glass protein nanoparticle distribution within the fibers were analyzed using SEM. Generally, plain nanofibers without protein encapsulation showed randomly oriented fibers with uniform size distribution in the range of 422 ± 33 nm for PEU and 581 ± 30 nm for PCL with smooth surface morphology (Figure 2(a) and 2(d)). However, the rhGH-SGnPs encapsulated fibers showed much rougher surface and less uniform size as a consequence of protein nanoparticle aggregation within the fibers (Figure 2(b), PEU, 542 ± 128 nm; Figure 2(e), PCL, 360 ± 118 nm). Besides, when incorporating the sugar stabilized protein nanoparticles into the polymer system, a less stable

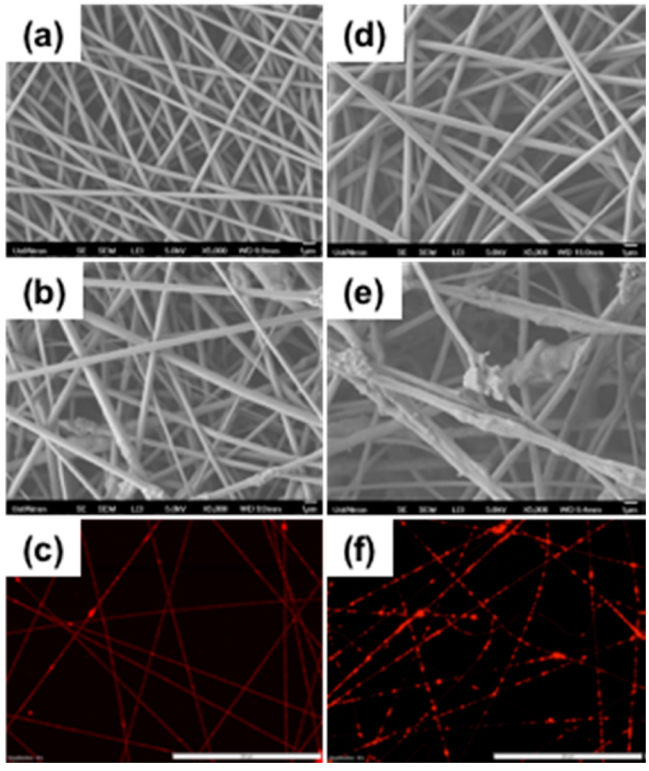

Figure 2. SEM and fluorescent images of polymer nanofibers: (a) plain PEU, (b) rhGH-SGnP loaded PEU, (c) RB-SGnP loaded PEU, (d) plain PCL, (e) rhGH-SGnP loaded PCL, and (f) RB-SGnP loaded PCL.

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electrospinning jet was observed, which may also lead to less uniform fiber diameters.

To study the protein nanoparticle distribution within the polymer fibers, fluorescent dye sugar glass nanoparticles (RB-SGnPs) were prepared and introduced into the corresponding polymer nanofibers. As confirmed by the fluorescent microscopy (Figure 2(c) and 2(f)), the fluorescent RB-SGnPs were found to be randomly dispersed throughout the nanofibers in an aggregate form. Furthermore, after comparing these two different polymer systems, we found that the aggregation of protein nanoparticles was much less within PEU nanofibers than within PCL ones. The aggregation may be due to the semicrystalline nature of the PCL as PEU is amorphous. The aggregation of protein nanoparticles in the polymer nanofibers may increase the chance of burst release when separation occurs between the polymer and protein-bearing phases.

Protein released from both rhGH-SGnP loaded PEU and PCL nanofibers was quantified using a BCA (bicinchoninic acid) protein assay. The cumulative rhGH release profile is shown in Figure 3. Sustained release of rhGH from both PEU and PCL nanofiber delivery systems was observed for up to 6 weeks with a modest burst release within the first 24 h (i.e., 7−10% for PEU and 13−15% for PCL). This long-term steady protein release is expected as a consequence of the slow degradation rates of PEU and PCL.[33,35,42,43] The initial burst release is highly likely attributed to the nanoparticles located on the surface of the fibers. Instead of being shielded by a significant amount of polymer to slow down the release, these nanoparticles have some direct exposure to the medium, and thus they are expected to be released rapidly. This behavior is illustrated in the inset of Figure 3. Additionally, aggregation of the protein nanoparticles within the polymer fibers could contribute to the initial burst release as well. We observed that the rhGH-SGnPs loaded PEU nanofibers, which had less protein nanoparticle aggregation than PCL surrogates, demonstrated lower burst release. Uncontrolled initial burst release is usually considered detrimental for sustained protein release systems, and here a reduced burst release was successfully obtained using our PEU delivery system. Another advantage of the PEU nanofiber delivery system is that the degradation of PEU does not cause local acidosis or adverse inflammatory reactions of surrounding tissues.[38,39] Therefore, this electrospun PEU delivery system has a great potential to be used as an implantable bioactive scaffold for local rhGH delivery.

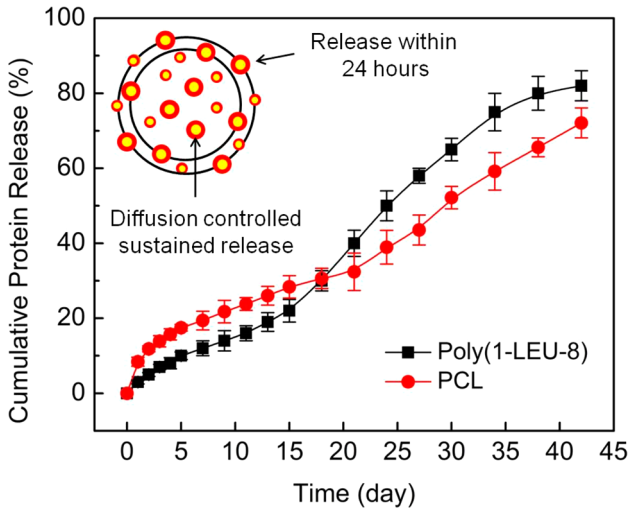

Figure 3. Sustained release profile of rhGH-SGnPs from PEU and PCL electrospun nanofibers within 6 weeks *in vitro* (inset: schematic presentation of rhGH-SGnPs distribution).

As mentioned above, growth hormone can degrade within a few days, and thus it is difficult to make a long-term effective delivery just by introducing it directly to a tissue scaffold. Here in our work, together with the successful sustained release of growth hormone from rhGH-SGnPs or rhGH-SGnPs encapsulated PEU and PCL nanofibers, their bioactivity was further detected using an Nb2 cell bioassay. The Nb2 cells are capable of cross-reacting with the bioactive growth hormone isoforms through their lactogenic receptors,[44] thus providing an effective approach to evaluate the biological activity of growth hormone.[45] As demonstrated in Figure 4, fresh commercial growth hormone is bioactive, and Nb2 cell proliferation was largely promoted compared with cell-only control as expected. In addition, statistically significant differences ($p < 0.01$) were found between control and rhGH-SGnPs, rhGH-SGnPs in PCL, or rhGH-SGnPs in PEU nanofiber groups, indicating the successful release of bioactive human growth hormone.

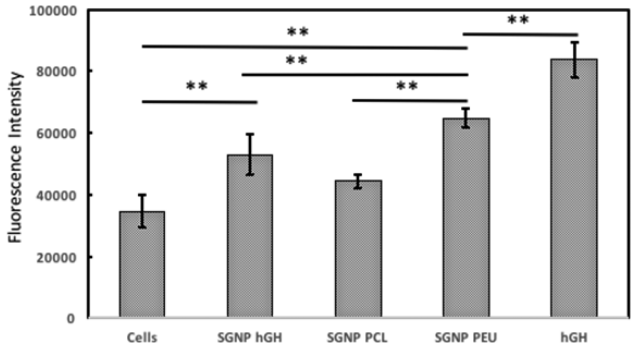

Figure 4. Bioactivity of released growth hormone from rhGH-SGnPS, rhGH-SGnPs in PCL, or rhGH-SGnPs in PEU nanofibers (each containing 6.25 μg/mL of rHGH) was detected by an Nb2 cell bioassay 48 h after cell seeding. Nb2 cells cultured with and without commercial growth hormone (1 μg/mL) were used as the positive and negative controls, respectively. (**: $p < 0.01$).

In summary, sugar glass nanoparticle stabilized rhGH was successfully incorporated into resorbable poly(ester urea) (PEU) nanofibers through electrospinning. The sugar nanoparticles were found to be randomly dispersed throughout the polymer fibers with some aggregation. A sustained release of bioactive rhGH from the electrospun PEU nanofiber mat for up to 6 weeks was observed without producing any acid degradation products, indicating its high potential to serve as an *in situ* long-term protein delivery system. Our future efforts will be optimizing a release profile and dosing for a clinical effect *in vivo*.

EXPERIMENTAL SECTION

Materials. 1,8-Octanediol (98%, Sigma-Aldrich), *p*-toluenesulfonic acid (99%, Fisher Scientific), triphosgene (98%, Alfa Aesar), sodium carbonate ($NaCO_3$, 99%, Fisher Scientific), dioctyl sulfosuccinate (AOT, 96%, Alfa Aesar), D-(+)-trehalose dehydrate (trehalose, 99%, Sigma-Aldrich), and PCL (poly(ε-caprolactone), 150 kDa, Scientific polymer products, Inc., Ontario, NY) were used as received. All solvents were HPLC grade, e.g., 2,2,4-trimethylpentane (isooctane), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), and analytical grade di-

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chloromethane (DCM), and dimethylformamide (DMF), 1,4-dioxane, and ethanol were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Recombinant human growth hormone (rhGH) was donated by the Akron Children's Hospital (Akron, OH). BCA assays were obtained from Pierce Biotechnology (Rockford, IL). Rhodamine B was purchased from Sigma-Aldrich (St Louis, MO).

Polymer Synthesis and Characterization. The poly(ester urea) monomer and polymer were prepared as described previously.[35] Poly(1-LEU-8): Mn = 77 kDa, Mw = 131 kDa; T_d = 272 °C, T_g = 37 °C.

Sugar Glass Nanoparticle Preparation and Characterization. SGnPs were prepared from inverse micelles of AOT in isooctane according to a published procedure with minor modification.[34] In brief, AOT (2.13 g, 0.0048 mol) was dissolved in 12 mL of isooctane in a 25 mL centrifuged tube to produce a 0.4 M solution. An aqueous solution containing protein with trehalose was then added dropwise while vortexing. The water:surfactant mole ratio (w = [H_2O]/[AOT]) was set to 15. The mass ratio of protein to trehalose was maintained at 1:200 (0.5 mg of protein in 100 mg of trehalose). After addition of the aqueous solution, the mixture was continuously vortexed for 2 min until a clear suspension was observed. The resulting inverse micelles were obtained by flash-freezing the clear suspension with liquid nitrogen. The frozen micelles were then lyophilized under vacuum for 3 days, followed by washing 5 times with isooctane. The resulting nanoparticles were resuspended in isooctane and stored in a desiccator at −20 °C until use. Fluorescent dye (Rhodamine B) loaded SGnPs were prepared using a similar method. Here in this work, sugar glass nanoparticles encapsulated with rhGH and rhodamine B are named as rhGH-SGnP and RB-SGnP, respectively. The morphology and size distribution of the nanoparticles were characterized by transmission electron microscopy (Philips TECNAI TEM) and dynamic light scattering (Brookhaven light scattering spectrometer, BI-200SM), respectively.

Electrospun Nanofiber Fabrication and Characterization. Plain PEU and PCL Nanofibers. A 10 wt % poly(1-LEU-8) polymer solution in HFIP and a 12 wt % PCL polymer solution in DCM:DMF = 4:1 (v/v) were electrospun to produce plain PEU and PCL nanofibers without protein loading.

PEU and PCL Nanofibers Loaded with rhGH-SGnPs or RB-SGnPs. To fabricate growth hormone or fluorescent dye nanoparticle encapsulated PEU fibers, poly(1-LEU-8) was initially dissolved in 1,4-dioxane at a concentration of 12 wt %. Growth hormone or fluorescent dye was added into the PEU polymer solution in the form of sugar glass nanoparticles. The rhGH-SGnPs or RB-SGnPs suspension in isooctane was centrifuged at 300g for 2 min. After discarding the supernatant, a mixture of 1,4-dioxane and EtOH was added to the precipitate. The reconstituted rhGH-SGnP or RB-SBnPs suspension was then transferred to the prepared PEU polymer solution. The resulting SGnPs-polymer suspension was vortexed to distribute the protein or dye nanoparticles uniformly throughout the polymer solution. The final polymer concentration was 7 wt % in 1,4-dioxane:EtOH = 2:1 (v/v) for electrospinning, and the loading level of growth hormone or fluorescent dye nanoparticles in the nanofibers was 1.6 wt %. PCL nanofibers were prepared similarly to those for PEU nanofibers, except that a mixture solvent of DCM:EtOH = 3:1 (v/v) was used instead for electrospinning.

The electrospinning setup included a syringe pump, a high voltage supply, and a collector covered with aluminum foil. A positive high voltage (10 kV) was applied to the polymer solution by the power supply, and the mixture solution was delivered through a 21 gauge blunt tip syringe needle at a constant flow rate of 3 mL/h to produce fine polymer nanofibers. The collecting distance between the syringe needle tip and the aluminum foil was 15 cm. The obtained polymer nanofibers were carefully peeled off from the aluminum foil and further dried for at least 12 h under vacuum to remove solvent residue. Fiber diameter, morphology, and distribution of sugar glass nanoparticles within the fibers were studied by scanning electron microscopy (JEOLJSM-7401F SEM) and fluorescence microscopy (OLYMPUS IX 81).

***In Vitro* Protein Release Study.** Approximately 90 mg of electrospun nanofibers encapsulated with protein SGnPs was soaked in 10 mL of PBS in a 20 mL glass vial. The glass vial was incubated at 37 °C in the presence of 5% carbon dioxide with mild stirring. At various time points, 0.15 mL of supernatant was retrieved from the vial followed by an equal amount addition of fresh medium. The released protein was quantified using a Micro BCA (bicinchoninic acid) Protein Assay Kit (Pierce, USA) according to the manufacturer's protocol. Absorbance at 562 nm was measured on a Synergy MX plate reader from BioTek. The protein release amount was calculated using an established standard curve of rhGH ranging from 0 to 250 μg/mL. All release experiments were conducted in triplicate.

Bioactivity of Released rhGH. The bioactivity of the released growth hormone was detected by an Nb2 cell bioassay according to the manufacturer's protocol. In brief, rat node lymphoma (Nb2) cell line (ECACC cell line, Sigma-Aldrich) was routinely cultured in 75 cm^3 nontreated tissue culture flasks for suspension cells in Fisher's medium supplied with 10 vol % of fetal bovine serum (FBS), 10 vol % of horse serum, extra Na-bicarbonate (0.075%), and 2-mercaptoethanol (2-ME, 50 μM) at 37 °C in a humidified incubator with 5% CO_2 and 95% air. Reduced cell replication rate was obtained by transferring cells from a high lactogen medium (with 10% FBS) to a lactogen-deficient medium (Fisher's medium with 1% FBS, 10% horse serum, and 50 μM 2-ME). After 24 h, cells were washed thoroughly by FBS-free medium (Fisher's medium with 10% horse serum, 50 μM 2-ME, and 10 mM HEPES) three times and then distributed to a 12-well plate (2 mL/well) at a concentration of 2.2×10^5 cell/mL. Sugar glass stabilized growth hormone nanoparticles (rhGH-SGnPs) and rhGH-SGnP loaded electrospun PEU nanofibers containing 12.5 μg of hormone were then added into the wells subsequently. Bioactivity of the released growth hormone was determined by the cell numbers 48 h after cell distribution into plates by a CyQUANT cell proliferation assay (Thermofisher Scientific) following the manufacturer's protocol. Nb2 cells cultured in FBS-free medium only and with 1 μg/mL of commercial hormone were used as the negative and positive controls, respectively.

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Notes

The authors declare no competing financial interest.

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