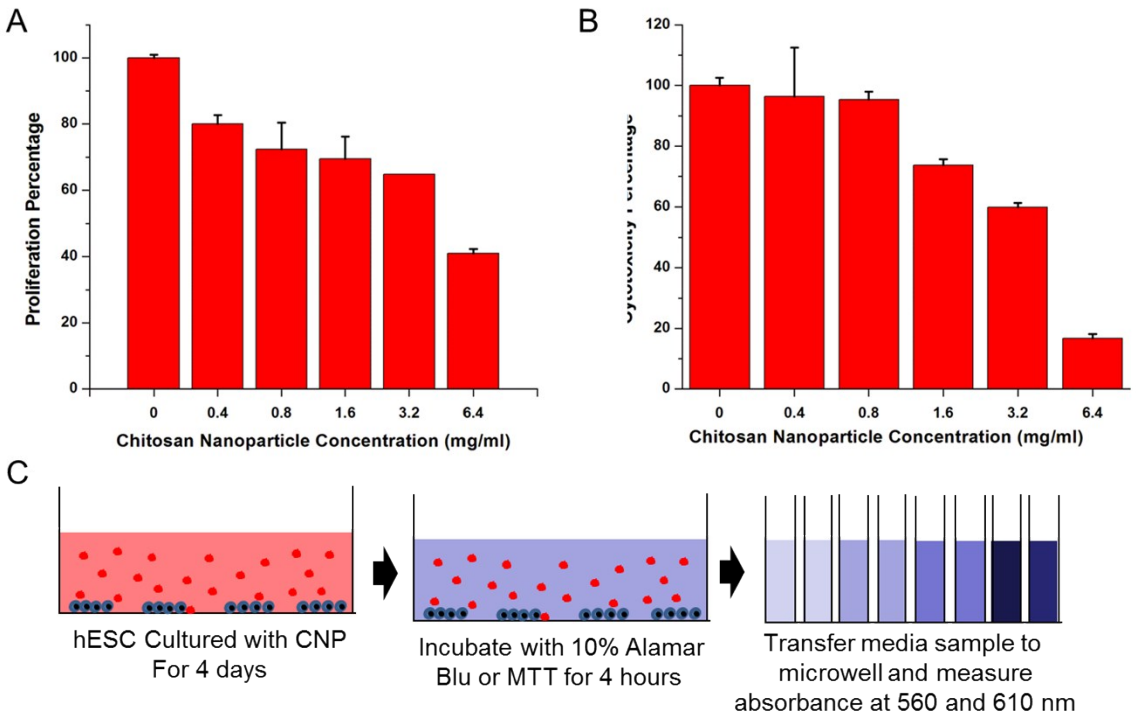
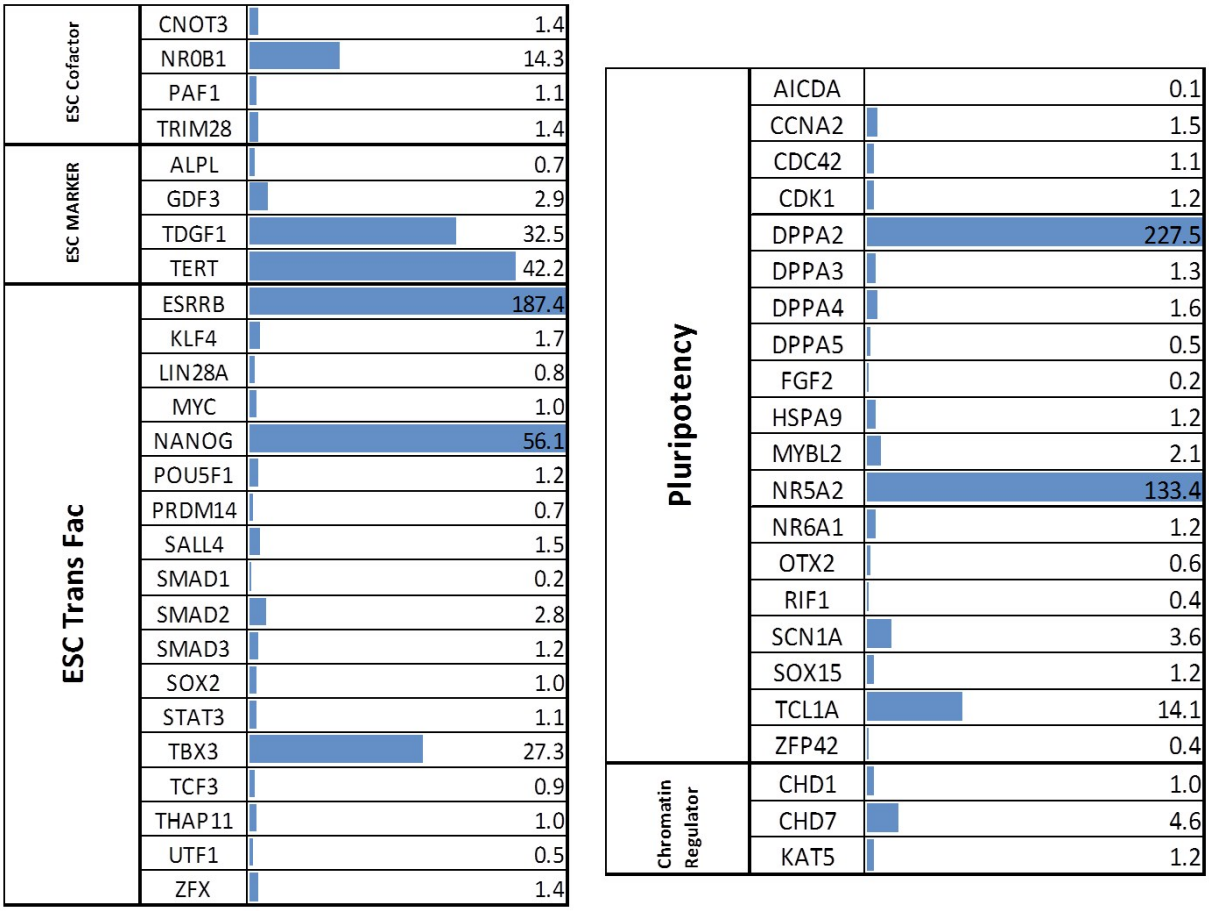


Supp. Figure 1: Human embryonic stem cells colonies in 2D maintain proliferation (A) and cell metabolic function (B) when chitosan nanoparticles are directly introduced through the media (C).

The hESCs were exposed to a broad range of CNP concentration: from 0.4 mg/ml up to 6.4 mg/ml CNP concentration in media. After 4 days exposure in culture, the proliferation rate was determined using AlamarBlue assay (Supp. Figure 1A). At a 0.4 mg/ml CNP concentration proliferation was 80% as compared to a control group of cells cultured without CNP exposure. At CNP concentrations up to 3.2 mg/ml the cell proliferation rate remained above 65% of control, however at 6.4 mg/ml CNP concentration there was a drastic decrease in cell proliferation with rate dropping to 40% that of control. In addition to proliferation, after 4 days of exposure to CNP in culture, the overall cellular metabolic activity was determined by MTT assay (Supp. Figure 1B).

At CNP levels of 0.4mg/ml and 0.8 mg/ml experimental, the cellular metabolic activity remained above 95%. At up to 3.2 mg/ml CNP concentrations, there was a decrease of cellular activity to 60-70% of control activity. There was a drastic decrease in cellular activity of hESCs exposed to 6.4 mg/ml concentration of CNPs to 20% that of control hESCs.



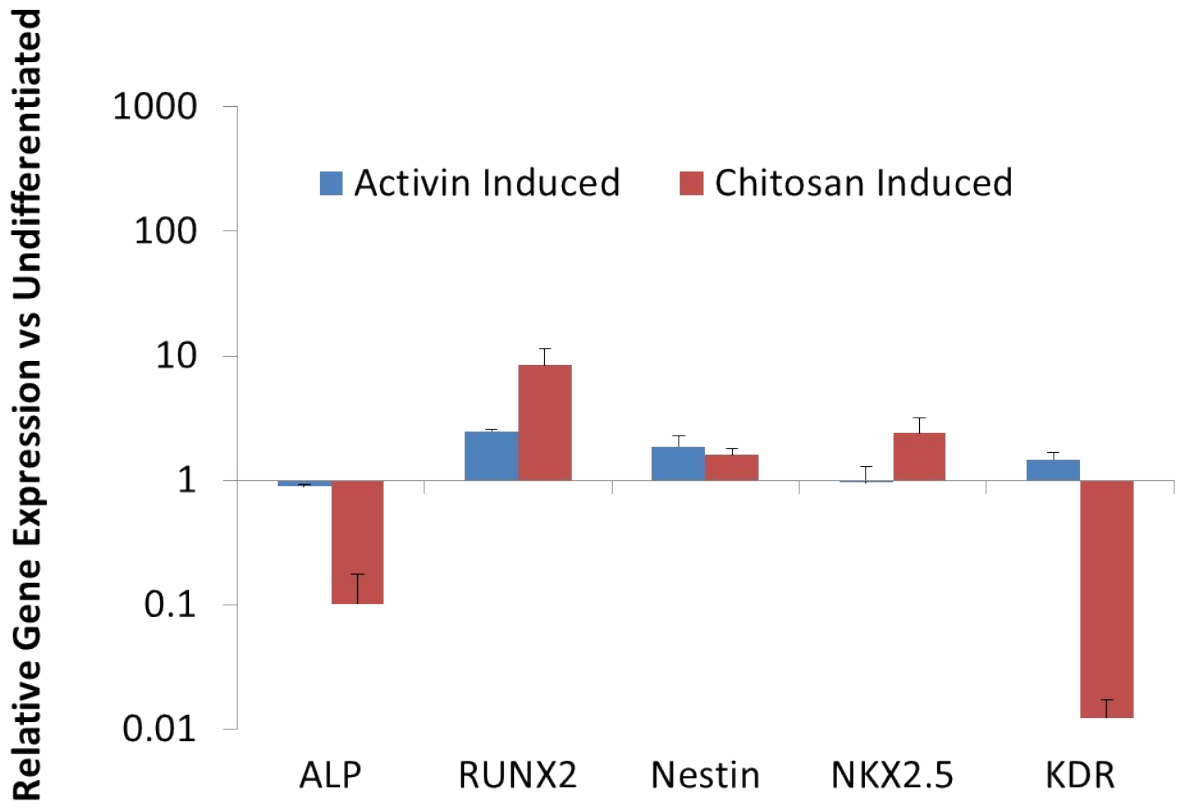
Supp. Figure 2: A PCR array specific for early human embryonic stem cell cofactors, markers, transcription factors, and pluripotency markers from Qiagen hESC array used to determine early lineage selection.

16% of the pluripotent markers and ESC transcription factors demonstrated a more than 10 fold increase in the CNP positive group, while almost 60% of pluripotent markers were downregulated

in the CNP-alginate hydrogel when compared to the alginate only control. Of these only *NR5A2* and *DPPA2* were highly upregulated. In the hESC specific gene markers there was a significant increase found for *TERT* and *TGF1*, while *ALPL* and *GDF3* were both downregulated.

Supp. Table 1

Chitosan to TPP Ratio (ml:ml)	**Particle Size (nm) by DLS**
1:2	3247 ± 742
1:1	1502 ± 634
2:1	220 ± 35
3:1	276 ± 24



Supp. Figure 3: Gene expression analysis of mature markers after chemically induced pancreatic maturation of hESC derived Definitive Endoderm (DE) cells generated from ActivinA and CNPEA. Gene expression markers were chosen from mature markers of mesoderm lineage (ALP, RUNX2), ectoderm lineage (Nestin), cardiac associated cells (NKX2.5), and hematopoietic stem cell derivatives (KDR). Minimal upregulation was noted across the negative controls when compared to the high upregulation of mature Beta cell genes from Fig. 5.

SUPPLEMENTARY MATERIAL AND METHODS

Chitosan Nanoparticle Formation

A 3.56 mg/ml chitosan (Sigma Aldrich) solution was prepared in 1.5% v/v acetic acid (Fisher Scientific) and titrated to a pH of 5.0 using 1M NaOH buffer. A 1.875 mg/ml tripolyphosphate (TPP) solution is slowly added to the chitosan solution under magnetic stirring to form CNPs. After formation the resulting suspension was centrifuged at 4,000 rpm for 40 minutes at 4 C. The supernatant was removed and the CNPs were washed with a 1.5% v/v acetic acid solution to remove excess chitosan. CNP particle size was measured in solution using dynamic light scattering for each CN to TPP ratio used (Table 1). FITC labeled CNPs were formed by 10 ml of methanol added to 10 ml of the 3.56 mg/ml chitosan in 1.5% acetic acid solution, followed by the addition of 5 ml of 2.0 mg/ml FITC (Sigma Aldrich). The chitosan was first precipitated by the addition of 0.5 M NaOH followed by centrifugation, then re-suspended in 1.5% Acetic Acid for further use.

CNP and hESC Encapsulation in Alginate

The evenly sectioned hESCs were manually scraped from the tissue culture plastic in the presence of experimental media. After centrifugation, cells were suspended at room temperature in 1.1% (w/v) low viscosity alginate (Sigma-Aldrich, St Louis, MO, USA) with 0.2 % (v/v) gelatin (Sigma

Aldrich). For co-encapsulation with CNP, 0.4 mg/ml of chitosan nanoparticles were dispersed in the alginate using sonication prior to the addition of the sectioned hESC colonies. The alginate solutions containing hESC colonies were added drop wise using a 22 gauge syringe into a magnetically stirred solution of 100 mM calcium chloride (Sigma-Aldrich) buffered with 10 mM HEPES (Sigma-Aldrich). After 5 minutes incubation in the calcium chloride bath, the microcapsules underwent 3 successive washes of phosphate buffered saline followed by a wash in DMEM:F12. Microcapsules were transferred to a 6-well tissue culture plate and the appropriate media was added until the microcapsules were completely submerged. For all 3D differentiation studies the base experimental media (DMEM:F12, B27 Supplement, 0.2%BSA) alone represented our spontaneous control, while the experimental groups were supplemented with human 100 ng/ml human Activin-A, 0.4 mg/ml CNP, or both (Fig 5G).

Harvesting Cell Colonies from Microcapsules

To collect hESC colonies from the alginate microspheres for further study a chelating solution was used to dissolve the alginate microcapsules. Entire microspheres containing cell colonies were incubated in a 100 mM EDTA (Sigma-Aldrich) solution in PBS for 5 minutes while under mechanical shaking and collected by centrifugation at 250 rcf for 4 minutes. If the alginate microspheres were not completely dissolved the previous step was repeated.

Cell Functionality Assays

To verify the viability of encapsulated hESC colonies immediately after encapsulation and after differentiation a commercially available LIVE/DEAD (Life Technologies) viability assay was used. Entire microcapsules containing hESC colonies were incubated for 25 minutes with 2 □M

ethidium homodimer-1 and 1 □M calcein-AM in DMEM:F12 at room temperature. The microcapsules were washed 3 times successively with PBS for fluorescent imaging.

The effect of hESC exposure to CNP on cell proliferation and cytotoxicity was initially determined in 2D with commercially available MTT (Life Technologies) and AlamarBlu (BioRAD) assay kits. hESC colonies cultured on matrigel coated tissue culture plastic were exposed to experimental media (DMEM:F12, B27 supplement, and 0.2% BSA) containing CNPs at concentrations ranging from 0.4 mg/ml to 6.4 mg/ml. After 4 days in culture, all CNPs were washed and fresh experimental DMEM:F12 was added to each well. The media was supplemented with 10% AlamarBlue or MTT solution as described in the kits. After incubation, 100 ul of the media was removed from each sample and placed in a 96-well plate. The absorbance at 570 nm and 600 nm was read with a plate reader and the percent proliferation or cytotoxicity as compared to hESCs without CNPs was calculated as described in the assay kits. The effect of 3D hESC encapsulation with CNPs was also investigated. After 4 days of encapsulation with and without CNPs the proliferation was tested with AlamarBlue as described above. The absorbance values were compared to controls tested immediately after encapsulation.

Quantitative Reverse-transcription Polymerase Chain Reaction

Gene analysis was performed using the ΔΔCt method (Supp) of calculating fold change in gene expression. For further gene analysis to isolate the CNP effect on early lineage commitment in hESCs the experimental groups were expanded to include hESCs encapsulated with CNP, hESCs encapsulated in alginate alone with human Activin-A introduced in the media, and a combination of both. After 4 days of differentiation RNA was again isolated using a Nucleospin RNA II kit

(Macherer-Nagel). The extracted RNA was used to synthesize cDNA by ImProm II Reverse Transcriptase System (Promega). Gene expression analysis was performed on each experimental group using quantitative real time RT-PCR analysis. The relative fold change of gene expression between a sample and the undifferentiated control population is given by equation 3.

$Ct_{marker\ gene} - Ct_{GAPDH} = \Delta Ct$ (1)

$\Delta Ct_{Experimental} - \Delta Ct_{Spontaneous\ Control} = \Delta\Delta Ct$ (2)

Relative fold change in marker gene = $2^{-\Delta\Delta Ct}$ (3)

Further Differentiation towards Pancreatic Progenitor and Mature Cell Stages

Encapsulated hESCs were exposed to either CNP or AN for 4 days to reach definitive endoderm. From this stage they underwent identical chemically induced maturation first towards the pancreatic progenitor stage and then towards a mature islet stage. A base media containing DMEM/F12 (Life Technologies), 0.2% bovine serum albumin (BSA), and 1X B27 supplement (Life Technologies) was used throughout differentiation. The first stage of differentiation from hESC to a definitive endoderm state lasted 4 days with base differentiation media supplemented with 100ng/ml Activin A or 0.4 mg/ml CNP with media changed daily. The second stage of differentiation from definitive endoderm to pancreatic progenitor involved base media supplemented with 0.2uM KAAD-cyclopamine for 4 days, with 2uM Retinoic Acid also added on the final 2 days. The finals stage of differentiation from PP to final maturation was performed with base media supplemented with 10uM nicotinamide for 9 days, with 30uM DAPT (SC) also added the final 7 days.

Immunostaining for Definitive Endoderm Protein Expression

Encapsulated cell colonies fixed with 4% formaldehyde for 30 minutes were collected from alginate encapsulation as previously described and suspended in Histogel (Thermo Scientific). After alcohol dehydration using a series of ethanol washes of increasing concentration the cell colonies were embedded in paraffin for sectioning and mounted on glass slides. Prior to further processing, a citrate buffer antigen retrieval step was performed. The cell colony sections were permeabilized with 0.1% Triton-X (Sigma) in PBS for 5 minutes, followed by a PBS wash. A one hour non-specific blocking step using 10% donkey serum in PBS was done before primary antibody incubation. The definitive endoderm specific primary antibodies used in this study were goat anti-SOX17 (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-FOXA2 (1:200 dilution, Santa Cruz). After primary antibody incubation overnight at 4 C the slides underwent three successive washes in PBS for 5 to 10 minutes each. Secondary antibody (either donkey anti-goat IgG-FITC or donkey-anti rabbit IgG-FITC, Santa Cruz) was introduced for 45 minutes at room temperature with a dilution of 1:200. The samples underwent three PBS washes of 5 minutes each followed by final mounting with hardening medium containing DAPI (Vectashield, Vector Laboratory). All fluorescent images were taken using Olympus IX-81flourescent microscope.

AFM Imaging and Nanoindentation

All AFM imaging and force indentation measurements were performed using the Bio-MFP-3D (Asylum Research). Samples were imaged using intermittent contact with a commercially available silicon nitride cantilever with a spring constant of 1.4 N/m (Veeco). AFM nano-

indentation measurements were taken using a silicon nitride cantilever (spring constant = 0.8 N/m) with a silica spherical probe (radius = 7 microns) attached.

Flow Cytometry

After 4 days, alginate encapsulated CNPs and cells were harvested from microcapsules as previously mentioned above. The collected cells and residual CNPs were plated on matrigel coated tissue culture plastic for 12 hours, followed by a PBS wash to remove CNPs that were not incorporated into the cells. The cell colonies were disassociated into single cells by accutase treatment for 5 minutes. Single cells were centrifuged for 4 minutes at 270 rcf to remove accutase and re-suspended in PBS. hESCs that were not exposed to CNPs were used as a control. Single cell fluorescent intensity and size were captured with an Accuri C6 Flow Cytometer.