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Genetically programmed superparamagnetic behavior of mammalian cells

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ABSTRACT

Although magnetic fields and paramagnetic inorganic materials were abundant on planet earth during the entire evolution of living species the interaction of organisms with these physical forces remains a little-understood phenomenon. Interestingly, rather than being genetically encoded, organisms seem to accumulate and take advantage of inorganic nanoparticles to sense or react to magnetic fields. Using a synthetic biology-inspired approach we have genetically programmed mammalian cells to show superparamagnetic behavior. The combination of ectopic production of the human ferritin heavy chain 1 (hFTH1), engineering the cells for expression of an iron importer, the divalent metal ion transferase 1 (DMT1) and the design of an iron-loading culture medium to maximize cellular iron uptake enabled efficient iron mineralization in intracellular ferritin particles and conferred superparamagnetic behavior to the entire cell. When captured by a magnetic field the superparamagnetic cells reached attraction velocities of up to 30 μm/s and could be efficiently separated from complex cell mixtures using standard magnetic cell separation equipment. Technology that enables magnetic separation of genetically programmed superparamagnetic cells in the absence of inorganic particles could foster novel opportunities in diagnostics and cell-based therapies.

1. Introduction

Although iron is an essential nutrient found in the active centers of enzymes and oxygen carrier proteins, it is highly reactive in its free Fe^{2+} form and can cause oxidative damage to cells (Henle et al., 1999; Sohn and Yoon, 1998). Facing the opposing challenges of having sufficient iron supply while preventing iron-induced detrimental effects, iron uptake and storage are tightly regulated in all species (Andrews et al., 2003; Muckenthaler et al., 2008; Thomson et al., 1999). Intracellular iron storage is managed by a 24-mer 12 nm protein shell known as ferritin which is capable of converting and storing up to 4500 free iron ions of the less toxic ferric (Fe^{3+}) form in its 8 nm cavity (Treffry et al., 1997). In mammals, iron storage is managed by two functionally distinct ferritin subunits, the light L-ferritin isoform, which enhances iron hydrolysis and mineralization, and the heavy H-ferritin variant, that carries the ferroxidase activity and is essential for uptake of free iron (Hempstead et al., 1997). In order to match iron abundance with ferritin, the expression of the storage proteins is under transcriptional and translational control of iron and iron-dependent factors (Muckenthaler et al., 2008). For optimal iron homeostasis iron storage must be coordinated to iron import. Dietary iron is absorbed as Fe^{2+} by the intestinal lumen and directly imported into the cytoplasm of duodenal enterocytes by the divalent metal ion transferase 1 (DMT1) which is expressed in the apical membrane (Tabuchi et al., 2002). Therefore, Fe^{2+} is either converted to Fe^{3+} and stored in ferritin particles or directly transported across the basolateral membrane by ferroportin, where it reaches circulation and binds to transferrin after oxidation to Fe^{3+} (Nelson et al., 2010). Transferrin and transferrin receptor are important iron-import components that are regulated in response to intracellular iron concentration in all the cells that require iron (Casey et al., 1989). The extracellular transferrin captures two Fe^{3+} ions and then binds to the transferrin receptor in the plasma membrane, which leads to receptor-mediated endocytosis. The iron is recovered due to the pH change after endosome–lysosome fusion. While the iron-free transferrin receptor complex is recycled to the plasma membrane, where transferrin is released to search for more iron, the internalized iron is converted to Fe^{2+} and transferred from endosomes to the cytoplasm by the DMT1. In the cytoplasm, the Fe^{2+} is either immediately used or stored in ferritin as Fe^{3+}. Since proteins themselves are unable to exhibit superparamagnetic behavior we theorized that protein–metal complexes such as ferritin could be used to program cells to become superparamagnetic.

Spatiotemporal manipulation of cells using magnetic fields has recently attracted a lot of attention as a cell-separation technology (Miltenyi et al., 1990; Nishida and Silver, 2012; Schlatter et al., 2001), for targeted lentiviral transduction (Weber et al., 2009),

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as contrast agents for magnetic resonance imaging (Franek and Dolnikova, 1991), for the detection of cancer cells in the blood (Racila et al., 1998), magnetic hyperthermia-based eradication of cancer cells (Balivada et al., 2010), and for tracking of stem cells (Jendelova et al., 2004) or leukocytes (Wu et al., 2007). Currently, most applications are based on inorganic magnetic particles with a diameter in the low micrometer range that are conjugated to streptavidin or antibodies for specific surface tagging of cells (Miltenyi et al., 1990; Racila et al., 1998). Nanoparticles can more efficiently respond to magnetic fields than ferritin and are readily taken up by mammalian cells (Arbab et al., 2004; Pawelczyk et al., 2009; Weber et al., 2009). However, ongoing safety concerns associated with nanoparticles and their potential to promote deleterious catalysis in the cytosol (Bae et al., 2011; Knaapen et al., 2002) have dampened the hope for economic production and easy-to-apply magnetic particles for diagnostics and therapy. Although magnetic separation on the basis of ectopic expression of ferritin has previously not been successful in mammalian cells, we have revisited the issue and succeeded in programming mammalian cells to show superparamagnetic behavior using ferritin-based nanoparticles.

2. Materials and methods

2.1. Vector design

Total RNA was isolated from HEK-293T cells (see below) with an RNeasy mini kit (Qiagen, Hombrechtikon, Switzerland) and used to produce a cDNA pool with MultiScribe™ Reverse Transcriptase according to the manufacturer's instructions (Applied Biosystems, Carlsbad, CA, USA). N-terminally HA-tagged human ferritin heavy chain 1 (HA-hFTH1) was PCR amplified from the cDNA pool using oligonucleotides OTK26 (5′-ATAAGAATGCTAGCCCCACCATG*TACCCATACGATGTTCCAGATTA-CGCT*GGCGGCGGCGGCatgacgaccgcgtccacctcg-3′; *Nhe*I site underlined, HA tag in italics, annealing sequence lower case) and OTK22 (5′-ATAAGAATGCGGCCGCGGATCCttagctttcattatcactgtctcc-3′, *Not*I site underlined, annealing sequence in lower case), restricted with *Nhe*I and *Not*I and cloned into the corresponding sites (*Nhe*I/*Not*I) of pcDNA3.1(+) (pTK8, P_{hCMV}-HA-hFTH1-pA_{bGH}). pTK14 (P_{hCMV}-HA-hFTH1-$IRES_{PV}$-EYFP-pA_{bGH}) was constructed by excising $IRES_{PV}$-EYFP from pCF76 ($P_{hEF1\alpha}$-$IRES_{PV}$-EYFP-pA_{bGH}, unpublished) with *Not*I/*Xba*I and cloning the fragment into the corresponding sites (*Not*I/*Xba*I) of pTK8. Human divalent metal transporter 1 (DMT1, GenBank ID: AB004857) expression vector pDMT1 (P_{hCMV}-DMT1-pA_{hGH}) was purchased from Origene (Rockville, MD, USA).

2.2. Cell culture and transfections

Human embryonic kidney cells (HEK-293T, ATCC: CRL-11268) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS; lot no. P231902; PAN Biotech GmbH, Aidenbach, Germany) and 1% penicillin/streptomycin solution (Sigma–Aldrich, Munich, Germany) at 37 °C in a humidified atmosphere containing 5% CO_2. HEK-293T were cotransfected using an optimized $CaHPO_4$-based protocol. In brief, 2×10^5 HEK-293T were seeded per well of a 6-well plate and (co-)transfected with a total of 3 μg DNA (for cotransfections equimolar plasmid ratios were used) diluted in 100 μl 0.5 M $CaCl_2$ solution and subsequently mixed with 100 μl 2× HBS solution (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.1). The DNA-containing solution was incubated for 20 min at 22 °C before it was added to the cells. To load cells with iron they were either incubated for 6 h in iron-incorporation buffer (20 mM HEPES, 130 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 100 μM nifedipine, pH 6.5) containing 5% FCS and 3 mM of ferrous ammonium sulfate or grown for 36 h in culture medium supplemented with 3 mM of ferrous ammonium sulfate, unless stated otherwise. The cells were counted and their viability was assessed using an electric field multi-channel cell counting device (Casy® Cell Counter and Analyser Model TT; Roche Diagnostics GmbH, Basel, Switzerland).

2.3. Prussian blue staining of iron-loaded ferritin

24 h after transfection, the monolayer cultures were fixed with 100% ethanol at 22 °C for 10 min and washed with PBS (500 ml; 4 g NaCl, 0.1 g KCl, 0.72 g Na_2HPO_4, 0.12 g KH_2PO_4, pH 7.4) and incubated for 5 min at 22 °C with freshly prepared 4% potassium hexacyanoferrate (II) trihydrate (Sigma–Aldrich) solution containing 10% HCl (Sigma–Aldrich). Alternatively, the cells were detached using trypsin (500 μl, PAN Biotech) and lysed on ice in 50 μl lysis buffer (0.14 M NaCl, 0.1 M HEPES, 1.5% [w/v] Triton X-100 and protease inhibitor cocktail [Sigma–Aldrich; cat. no. P8340-1ML], pH 7.4). The cell lysate was centrifuged at $14{,}000 \times g$ for 20 min at 4 °C to remove cell debris and 20 μl of the supernatant was mixed with 1 μl of a 100 mM aqueous ferrous ammonium sulfate solution (Sigma Aldrich; cat. no. 09719-250G) and 10 μl of native gel loading buffer (10 ml; 1.25 ml 0.5 M Tris–HCl pH 6.8, 3 ml glycerol, 0.2 ml 0.5% (w/v) bromophenol blue, 5.55 ml deionized H_2O). The samples were resolved on a 6% polyacrylamide gel (PAGE) using 5 μg horse spleen ferritin (Sigma–Aldrich) as loading control. To visualize iron-loaded ferritin, the gel was then stained for 5 min at 22 °C in freshly prepared 4% potassium hexacyanoferrate (II) trihydrate (Sigma–Aldrich) solution containing 10% HCl (Sigma–Aldrich).

2.4. Western blot analysis

The cells were lysed (see above) and the extract resolved on a 12% SDS-PAGE as described above (1% (w/v) SDS added in loading buffer and running buffer). The proteins were electroblotted (Trans-Blot® SD, Bio-Rad, Reinach, Switzerland) onto a PVDF membrane (Immobilon®-P, Millipore, Billerica, MA, USA) which was blocked with 5% milk in TBST (TBS; 20 mM Tris, 137 mM NaCl, pH 7.4, containing 0.05% Tween-20; Axon Lab AG, Baden-Dättwil, Switzerland) for 1 h at 22 °C and incubated over night at 4 °C with a mouse anti-ferritin H chain polyclonal antibody (1:1000; Abnova, Taipei, Taiwan; cat. no. H00002495-B01, lot no. 08088 WULZ) or a rabbit anti-actin polyclonal antibody (1:1000; Sigma–Aldrich; cat no. A2066, lot no. 061M4871). The membrane was then washed three times in TBST and incubated with a horseradish peroxidase-coupled anti-mouse IgG (1:4000; Santa Cruz Biotechnology; Santa Cruz, CA, USA, cat no. SC-2306, lot no. E279) or a horseradish peroxidase-coupled anti-rabbit IgG (1:4000; AbD Serotec, Düsseldorf, Germany; cat no. STAR54). ECL-Plus Western blot detection reagents (Amersham, Piscataway, NJ, USA) were used for chemiluminescence-based signal detection performed with a Chemilux CCD camera (ImageQuant™, LAS 4000 mini, GE Healthcare, Switzerland).

2.5. Quantification of intracellular iron content

Intracellular iron concentration was quantified using a modified protocol adapted from a previous study (Rad et al., 2007). In brief, 1×10^6 cells were incubated for 6 h in iron-incorporation buffer (see above), washed three times with 1 ml of cell culture media (see above) to remove extracellular iron, detached using 0.5 ml trypsin and collected in 1.5 ml Eppendorf tubes. The cells were centrifuged at $1200 \times g$ for 1 min, the supernatant was discarded and the cell pellet was dried for 30 min at 95 °C. Dried cells were resuspended in 400 μl of 5 M HCl and incubated at 60 °C for 2 h to dissolve