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Functional behavior and gene expression of magnetic nanoparticle-loaded primary endothelial cells for targeting vascular stents

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Abstract

Aim—To assess functional competence and gene expression of magnetic nanoparticle (MNP)-loaded primary endothelial cells (ECs) as potential cell-based therapy vectors.

Materials & methods—A quantitative tube formation, nitric oxide and adhesion assays were conducted to assess functional potency of the MNP-loaded ECs. A quantitative real-time PCR was used to profile genes in both MNP-loaded at static conditions and *in vitro* targeted ECs.

Results—Functional behavior of MNP-loaded and unloaded cells was comparable. MNPs induce expression of genes involved in EC growth and survival, while repress genes involved in coagulation.

Conclusion—MNPs do not adversely affect cellular function. Gene expression indicates that targeting MNP-loaded ECs to vascular stents may potentially stimulate re-endothelialization of an implant and attenuate neointimal hyperplasia.

Keywords

endothelial integrity and functional potency; gene expression; in-stent restenosis; magnetic cell delivery; magnetic nanoparticles; primary endothelial cells; re-endothelialization

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Heart disease is the leading cause of morbidity and mortality in industrialized nations, and the epidemic is rapidly expanding to include the developing world [1]. Recently, it has been reported that cardiovascular related diseases account for one of every three deaths in the USA, and each day more than 2200 Americans die of cardiovascular diseases (CVDs) [2]. Many studies in humans and mice suggest that endothelial dysfunction is a cardinal feature of several CVDs [3]. Persistent hypertension and myocardial infarction perturb endothelial function and homeostasis, and endothelial dysfunction is intimately associated with the pathogenesis of both atherosclerotic lesions and neointima (NI) formation. Therefore, significant efforts have been devoted to developing pharmacological and mechanical strategies to protect endothelium homeostasis and/or accelerate its repair after vascular injury.

Among these strategies, percutaneous coronary intervention (PCI) whereby a metallic scaffold device (i.e., stent) is the most commonly utilized approach to relieve obstructive atherosclerotic lesions. However, long-term effectiveness of PCI is hampered by NI formation, resulting in renarrowing of the stented artery, a process known as in-stent restenosis (ISR) [4]. Activation of platelets, macrophages and leukocytes as well as induction of smooth muscle cell (SMC) migration, proliferation and deposition of extracellular matrix (ECM) is known to contribute to ISR [5,6]. Although drug-eluting stents reduce the incidence of ISR, the associated impaired re-endothelialization (RE) and the resultant risk of 'late stent thrombosis' due to the drug and/or the polymer in these devices remain formidable obstacles [6].

In an alternative approach, early restoration of endothelial integrity was achieved through local administration of VEGF resulting in reduced proliferation of intimal vascular SMCs, providing indirect evidence to support a role of endothelial integrity in suppressing intimal hyperplasia [7–9]. These studies suggest that targeting endothelial cells (ECs) to vascular stents could provide long-term therapeutic benefit in preventing ISR through accelerated RE and subsequent repair of injured blood vessels.

Our group is developing a strategy to target ECs to vascular stents utilizing magnetic forces [10]. In this approach, 'therapeutic' cells are loaded with magnetic nanoparticles (MNPs) to become responsive to a magnetic force. An externally applied uniform magnetic field across the stent magnetizes the stent's struts and generates a magnetic field gradient on the stent's surface. The same uniform magnetic field also magnetizes MNP-loaded ECs delivered near the stent, resulting in magnetically driven targeting of ECs to the stent's surface. The feasibility of this concept has been shown by our group both *in vitro* and *in vivo* using model bovine aortic ECs (BAEC) in outbred Sprague–Dawley rats [10]. Currently our group is conducting a long-term therapeutic efficacy study in rats. Rats are one of the smallest research animals that have been successfully used in a carotid artery stent angioplasty model [10–12]. The use of autologous cells in the long-term therapeutic efficacy studies is a preferable strategy to eliminate immune rejection of the targeted/implanted cells by the host. However, rats are too small for autologous EC transplantations. Therefore, our current therapeutic efficacy study is conducted in Lewis inbred rats for isogenicity and better acceptance of the nonautologous, but isogenic cell transplants. To this end, we isolated

primary rat aortic ECs (RAECs) to generate a working stock of isogenic cells for stent targeting experiments.

Because endothelial function plays a vital role in inhibiting NI formation after stent implantation and subsequent cell targeting to the stent, gene expression and functional behavior of the MNP-loaded RAECs could be critical for achieving successful RE and eventual prevention of ISR. However, the effects of MNPs on EC function and gene expression remain elusive, and a clear understanding of any significant alteration in these properties is a prerequisite for the future implementation of the cell targeting strategy in the context of vascular application. Motivated by this need, we conducted this study to assess endothelial integrity, functional behavior and expression changes of genes involved in endothelial growth and survival along with genes important for prevention of NI in primary RAECs loaded with MNPs at static conditions and targeted to a metal mesh *in vitro*.

Materials & methods

Isolation of primary RAECs

The study was performed with the approval and according to the guidelines of the Institutional Animal Care and Use Committee of the Drexel University, College of Medicine, PA, USA. After dissection, rat aorta was cannulated with 4–0 monofilament ligature and tied at the distal end making sure that the vessel is completely included into the tie. Then the vessel was carefully inverted by pulling on the ligature. The proximal end was covered with a thin sterile piece of rubber and tied with 4–0 ligature on the top of the rubber cap for complete isolation of the vessel ends. Capping of vessel ends is necessary to prevent coisolation of other non-EC types. Then the inverted vessel was incubated at 37°C for 15 min in 1.0 U/ml of Liberase™ (Roche Applied Science, IN, USA), rinsed in bare endothelial basal medium DMEM and transferred to DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. The vessel was incubated for 7 days without changing the medium. Passage zero ECs detached from the vessel lumen formed colonies along the aorta margins. The aorta then was removed and the cells propagated in culture for few passages. In our experiments we used primary ECs at passages 7 to 9.

Immunocytochemistry

RAECs were seeded on collagen precoated 35 mm MatTek glass bottom dishes (MatTek Corp., MA, USA) to reach 80% confluence at the time of staining. The cells were fixed with 4% formaldehyde (Thermo Fisher Scientific Inc., MA, USA) in phosphate-buffered saline (PBS) pH 7.4 for 15 min at room temperature. The cells were then permeabilized with 0.25% Triton X-100 in PBS for 15 min, washed in PBS three times for 5 min each and blocked with 5% bovine serum albumin (BSA; freshly prepared in PBS) for 2 h. Primary and secondary antibodies were diluted in 1% BSA in PBS and incubated on ice for 1 h and overnight, respectively. Immunofluorescence was photographed by Axiovert 40 CFL Microscope (Carl Zeiss, NY, USA) and analyzed using dry sample.

Primary antibodies were anti-CD31 (Abcam, Cambridge, UK; dilution: 1:50), anti-Tie-2 (C-20) (Santa Cruz Biotechnology, Inc., TX, USA; dilution: 1:50), anti-VE-cadherine (Santa

Cruz Biotechnology, Inc., TX, USA; dilution 1:25), anti-alpha smooth muscle actin antibody (Abcam; dilution: 1:400) and secondary antibodies were donkey anti-mouse IgG H&L (DyLight® 550; Abcam; dilution: 1:200), Goat anti-Rabbit IgG H&L (DyLight 550®; Abcam; dilution: 1:200), Goat anti-Rabbit IgG H&L (DyLight® 488; Abcam, dilution: 1:125). Cell nuclei of the fixed cells were visualized by staining with 4',6-diamidino-2-phenylindole, DAPI (Life Technologies, NY, USA).

Flow cytometry analysis of endothelial markers

For flow cytometry analysis, 1.5×10^6 cells per sample were fixed for 10 min with 2% formaldehyde (Thermo Fisher Scientific Inc.) in PBS. After centrifugation, the cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min and blocked with 5% BSA (Sigma-Aldrich, MO, USA). Primary antibody was diluted in 5% BSA in PBS and incubated with cells for 30 min on ice in the dark. The cells were then washed with PBS and centrifuged. Secondary antibody was diluted in 5% BSA in PBS and incubated with cells for 30 min on ice in the dark. After washing and centrifugation cells were resuspended with PBS and cell labeling was monitored using BD Accuri™ C6 Flow Cytometer (BD Biosciences, CA, USA), counting 10,000 events per sample.

Primary antibodies were anti-CD31 antibody (Abcam, dilution: 1:20), anti-alpha smooth muscle actin antibody (Abcam, dilution: 1:200), anti-Tie-2 (C-20; Santa Cruz Biotechnology, Inc., dilution: 1:40) and secondary antibodies were goat polyclonal antibody to mouse IgG1-gamma chain (Cy5®; Abcam, dilution: 1:200) for both CD31 and α -SMA, Goat anti-Rabbit IgG H&L (DyLight® 550; Abcam dilution: 1:100). Nonspecific fluorescence was assessed by incubation with isotype matched conjugated antibodies (dilution: same as primary antibody). For CD31 and α -SMA, isotype control was mouse IgG1, kappa monoclonal (MG1-45; Abcam). For Tie-2, isotype control was normal rabbit IgG (Santa Cruz Biotechnology, Inc.).

Acetylated low-density lipoprotein uptake assay

ECs are known to take up acetylated low-density lipoprotein (AcLDL) by receptor-mediated endocytosis [13]. The LDL uptake assay was conducted using RAECs, human hepatocellular liver carcinoma cell line (HepG2) as positive control and human mammary gland/breast cancer cell line (MCF7) as a negative control.

Cells were seeded on collagen precoated 35-mm MatTek glass bottom dishes (MatTek Corp., Ashland, MA, USA) and grown to a confluence. An LDL from human plasma, acetylated, Alexa Fluor® 488 Conjugate (Alexa Fluor 488 AcLDL; Life Technologies, USA) was incubated with cells for 4 h at 37°C at a concentration of 10 µg/ml. After incubation with AcLDL, the cells were washed with warm PBS three times and fixed with 4% formalin for 15 min at room temperature. The uptake of AcLDL by cells was assessed by fluorescent microscopy.

Preparation of nanoparticles & cell loading

Poly(lactic acid) (PLA)-based MNPs were prepared by the modified emulsification-solvent evaporation method as described elsewhere [14,15]. The particles contained $48.2 \pm 1.32\%$

(w/w) magnetite and were fluorescently labeled by BODIPY® 564/570 nm (Life Technologies, USA). The MNPs had a mean hydrodynamic diameter of 278 ± 1.62 nm and ζ -potential of -14.4 ± 0.34 mV. Magnetization of the MNPs measured at 5 kOe was 24.6 ± 1.22 emu/g of composite. Full physicochemical characterization of MNPs can be found in [16].

For cell loading, RAECs were seeded on clear-bottom 12-well plates (BD Biosciences) using MCDB 131 medium supplemented with 5% fetal bovine serum, EGF (10 ng/ml), Hydrocortisone (1 μ g/ml) and L-Glutamine (10 mM). 20 μ l (an equivalent of 0.736 mg) of the original MNP suspension were dispersed in 10 ml of the cell culture medium and filtered through a Millipore syringe driven filter with 5.0 μ m pores (Millipore Corporation, MA, USA). The MNP suspension was given to cells in the volume of 2 μ l (0.0736 mg) of MNPs per 300,000–350,000 cells (a given dose of ~ 100 pg magnetite/cell for 48% w/w MNPs) and incubated at 37°C for 24 h on a 96-well magnetic separator with an average surface field of 500 Gauss and field gradient of 32.5 T/m as a magnetic field source (LifeSep 96F, Dexter Magnetic Technologies, IL, USA). The cells were washed three times with PBS to remove particles not associated with cells. Then cells were trypsinized and resuspended in a cell culture medium for further RNA isolation, tube formation assay and magnetic capture experiments. After 24 h the magnetite content within RAECs was found to be 25.3 ± 0.75 pg magnetite/cell as quantified by the method described elsewhere [14]. Briefly, after loading with MNPs the cells were rinsed three times with PBS, trypsinized and counted. Cell suspensions then were centrifuged and the remaining pellet was digested with radioimmunoprecipitation assay buffer (RIPA) cell lysis buffer (Thermo Fisher Scientific Inc.) for 30 min at room temperature. After the centrifugation step, the iron content was determined spectrophotometrically (using Synergy™ 4 plate reader, BioTek Instruments, Inc., VT, USA and ultraviolet (UV) compatible 96-well plates (BD Biosciences) in 1N hydro-chloric acid ($\lambda = 335$ nm) against a standard curve after MNP degradation with 1N aqueous sodium hydroxide (90°C, 30 min) and dissolution of the iron containing precipitate in the acid solution.

***In vitro* cell-capture experiments**

In an *in vitro* cell-capture experiment, MNP-loaded RAECs ($3-4 \times 10^6$) circulated in a closed-loop system, including a magnetizable stainless-steel mesh, at a flow rate of 30 ml/min. A homogeneous magnetic field of 1200 Gauss was applied by passing an electrical current through serially connected solenoid coils with iron cores (45 mm in diameter) placed at both sides of a mesh positioned in a flow chamber of a model loop-circulatory system. The magnetic field strength was measured by a 410 hand-held gaussmeter equipped with transverse probe (Lake Shore Cryotronics, OH, USA). The cells captured on the mesh during 1 h of magnetic field application were imaged by fluorescent microscopy tracking either nanoparticles or live cells stained with CellTrace™ Calcein Green, AM (Life Technologies, USA). For RNA isolation the cells were isolated from the mesh by trypsinization, washed with the cell culture medium, centrifuged and frozen until further handling

Tube formation assay

MatrigelTM matrix (BD Biosciences) was thawed out overnight at 4°C on ice. Precooled plates, tips and tubes were used to dispense 30 µl of the MatrigelTM into the 96-well plate (BD Biosciences) placed on ice. To avoid air bubbles within the matrix, the plate was centrifuged at 2000 rpm for 10 min in a precooled centrifuge (4°C) without using breaks. Then the matrix was cured by incubation for 30 min at 37°C. Nonloaded and MNP-loaded RAECs suspended in MCDB 131 medium were seeded on a cured matrix at a density of 45,000–47,000 cells/cm². Different stages of tube formation were visualized at 4, 8 and 12 h using Axiovert 40 CFL Microscope (Carl Zeiss, NY, USA).

Wimasis WimTube image analysis software, the beta version (Wimasis, Munich, Germany), was used to quantitate various parameters in the tube formation assay, including number of tubules; number and mean number of junctions; tubule area (%); total, mean and standard deviation of tubule length; number of independent tubules and net characteristics (number of loops, mean perimeter loop and number of nets). The image analysis process was automated and involved filtering, segmenting, object recognition and data processing.

Quantitative real-time PCR array

Total RNA from nonloaded and MNP-loaded RAECs either at static or *in vitro* flow conditions was extracted using the RNeasy Mini Kit[®] (Qiagen, CA, USA) with DNase digestion. A total of 0.5–1 µg of RNA was reverse transcribed with the RT² First Strand Kit (SABiosciences, Qiagen) to cDNA. cDNAs were analyzed using Rat Endothelial Cell Biology RT² ProfilerTM PCR array (Qiagen), containing 84 genes involved in EC angiogenesis, vasoconstriction and vasodilation, inflammatory response, apoptosis, cell adhesion, coagulation and platelet activation according to the manufacturer's instructions. The expression data were analyzed through the free web module of SABiosciences, and the relative expression of each target gene was normalized using five 'housekeeping' genes. Primers of the 'housekeeping' genes were supplied in the PCR array plate and calculated according to the web module instructions. The fold changes in differential gene expression from three separate experiments with p-values were calculated and presented. A complete list of the genes included in the array can be found in manufacturer's manual.

Cell adhesion & nitric oxide quantification assays

Cell adhesion assay was performed using CytoSelectTM 48 well cell adhesion assay ECM array (Cell Biolabs Inc., CA, USA) per manufacturer's instruction. The total amounts of nitric oxide (NO) produced by MNP-loaded and nonloaded cells were quantified using colorimetric NO assay kit (Oxford Biomedical Research, MI, USA). The NO amounts were normalized to the total protein content in each cell sample to enable comparison of NO levels per cell.

Statistics

Statistical analyses were performed with GraphPad Prism version 5.03 for Windows (GraphPad Software Inc., CA, USA). Results are expressed as mean ± SEM from at least three independent experiments. Differences in the tube formation parameters and adhesion

for MNP-loaded and nonloaded RAECs were evaluated using a two-tailed, unpaired Student's t-test. Differences were considered significant at $p < 0.05$.

Results

Characterization of RAECs

We have isolated a pure culture of primary ECs from rat aorta (RAECs). These cells display characteristic EC cobblestone morphology (Figure 1A). Functionally, RAECs demonstrated positive uptake of AcLDL (Figure 1B). Consistently, HepG2 cells (positive control) were positive for AcLDL uptake, while MCF7 cells (negative control) showed negligible AcLDL uptake.

The endothelial phenotype of these cells was confirmed by immunostaining of specific endothelial markers such as VE-Cadherin, CD31 and Tie-2 (Figure 1C–E). RAECs were negative for SMC marker α -actin per immunofluorescence (Figure 1F). Flow cytometry indicates a highly pure EC population with 98% CD31 and 90% Tie-2 positive cells (Figure 1G & H). Isotype control antibodies were used as a negative control for flow cytometry analysis. Flow cytometry also proved that RAECs were negative for SMC marker α -SMA (Figure 1I). The RAECs also demonstrated efficient internalization of MNPs containing 25.3 ± 0.75 pg magnetite/cell per spectrophotometry. This level of MNP loading confers cells sufficient magnetization and enables their efficient capture to magnetizable implants with no apparent toxicity as was previously shown [17]. Flow cytometry showed a distinct population of MNP-loaded cells, indicative of homogeneous cell labeling (Figure 2A). Furthermore, this analysis shows that 96% of the RAECs are loaded with MNPs (Figure 2A) as also qualitatively evidenced by fluorescent microscopy (Figure 2B).

Tube formation assay

We evaluated functional behavior of MNP-loaded RAECs in *in vitro* tube formation assay using Matrigel. Both nonloaded and MNP-loaded RAECs were plated on Matrigel, a solubilized basement membrane extract from the Engelbreth-Holm-Swarm (EHS) mouse tumor, which is rich in basement membrane proteins. The major matrix components are laminin, collagen I, entactin and heparan sulfate proteoglycan. The matrix also contains growth factors, matrix metal-loproteinases (MMPs [collagenases]) and other proteinases (plasminogen activators [7]), as well as several undefined compounds [18,19]. Figure 3A shows that both nonloaded and MNP-loaded RAECs form a polygonal network at 4 h followed by formation of a stabilized honey comb at 8 h, and finally established network structure with lumens at 12 h. We observed that the tubular network persists up to 16 h. Then, usually after 24 h, the cells gradually undergo apoptosis and tubes detach from the matrix and break apart. The dynamics of tube formation by RAECs was validated using human umbilical vein endothelial cells as a positive control, which formed comparable structures. The observed tube formation dynamics clearly demonstrate the functional potency of RAECs after loading with MNPs. Further quantitative analysis of tube formation by nonloaded and MNP-loaded cells showed that the number of tubes and loops, branching points and covered area were similar in both nonloaded and MNP-loaded RAECs (Figure 3B). Some of the parameters (number of tubes at 4 and 12 h, number of loops at 4 h and

percent of covered area at 12 h) showed enhancement with statistical significance ($p < 0.05$) in MNP-loaded RAECs in comparison with nonloaded cells (Figure 3B). However, the enhancement in each case was marginal showing only 5 and 19.6% increase for number of tubes achieved at 4 and 12 h, respectively, while number of loops showed enhancement of 20% at 4 h and percent of covered area increased by 18.7% at 12 h for MNP-loaded RAECs (Figure 3B). This quantitative evaluation indicates on functional potency of MNP-loaded RAECs.

Adhesion & NO assays

Assessment of adhesion properties of MNP-loaded RAECs is also an important step in understanding their potential to repair injured endothelium *in vivo*. Adhesion assay using an extracellular matrix (ECM) array confirmed no alteration in adhesion on different biological substrates with MNP-loaded RAECs (Figure 4A).

NO production profile was comparable for non-loaded and MNP-loaded cells at two nanoparticle doses (25 and 12.5 pg magnetite/cell). The cumulative NO production linearly increased over a period of 3 days for all studied groups with no statistically significant difference at each time point (Figure 4B).

Gene-expression profile of MNP-loaded RAECs at static conditions

Because EC function plays a central role in prevention of thrombosis initiating a cascade of events leading to restenosis, assessment of endothelial gene expression after MNP-loading and targeting is essential. The Rat Endothelial Cell Biology RT² ProfilerTM PCR Array was used to profile the expression of 84 genes related to EC biology. This array includes genes involved in angiogenesis, inflammatory response, apoptosis, cell adhesion, coagulation and platelet activation. In static conditions, among these 84 genes, 76 genes showed changes in regulation of less than twofold. A total of eight genes showed up or downregulation more than twofold with significant p-value (<0.05) in separate PCR array experiments from three biological replicates (Table 1).

We were particularly interested in assessing the genes involved in inhibiting intimal hyperplasia as neointimal thickening following vascular injury is a major contributing factor for ISR [4]. A normal functional endothelium suppresses intimal hyperplasia by inhibiting thrombus formation and inflammation, the initial triggers of intimal hyperplasia, and by reducing SMC proliferation and migration [5]. The endothelium maintains an antithrombotic environment on its luminal surface by secreting factors such as NO and prostaglandin I₂, which inhibit platelet activation, adhesion and aggregation [20]. Interestingly, the *Nos3* gene (also known as *eNos*), which is responsible for synthesis of NO, was upregulated twofold in the MNP-loaded RAECs. The expression level of *Ptgis* gene, encoding the enzyme responsible for synthesis of prostaglandin I₂, a potent vasodilator and inhibitor of platelet aggregation, remained unchanged indicating on MNP-loaded RAECs' capability to maintain anti-thrombotic condition as normal nonloaded EC cells. ECs also maintain anticoagulatory and antifibrinolytic activity by secreting factors such as heparin, protein C and S, and tPa (Plat) [20]. Genes related to protein C and S also encode thrombomodulin (Thbd). Thbd is a cofactor of thrombin-induced activation of protein C in anticoagulant pathway, while protein

S functions as a cofactor to protein C. In our study, genes encoding for Thbd and tPa (Plat) remained unchanged in MNP-loaded cells.

Endothelium inhibits SMC growth by acting as a selectively permeable barrier protecting against circulating growth factors. By secreting factors such as NO, TGF- β , heparin and heparin-like molecules, ECs also preserve SMCs in a quiescent state [21,22]. As stated earlier, in MNP-loaded cells the *Nos3* (*eNos*) gene showed twofold upregulation, whereas TGF- β expression level did not change, indicating no alteration in MNP-loaded cells' potential to prevent SMC migration and proliferation.

Table 1 lists the genes that displayed a significant level of alteration. Three genes showed upregulation with more than twofold expression change. These genes were *Agt*, *Cxcr5* and *Nos3*. Five genes showed down-regulation with more than twofold changes and they are *Edn1*, *F3*, *Flt1*, *Serpine1* and *Thbs1*. Among these five, two genes (*Edn1* and *Thbs1*) showed more than fourfold change, while one gene (*Serpine1*) showed more than sixfold expression changes. We discuss in detail how changes in expression of these genes may be of benefit for our final goal of re-endothelialization, restoration of endothelial function and prevention of intimal hyperplasia.

Gene-expression profile of RAECs targeted *in vitro*

Because our final goal is to target MNP-loaded ECs to a magnetizable stainless steel stent *in vivo*, we examined the gene-expression profile of the MNP-loaded RAECs targeted to a stainless steel mesh in a model flow loop system in the presence of a uniform magnetic field as described elsewhere [10]. A homogeneous field is used to remotely and transiently magnetize the implant. This magnetization generates a magnetic gradient field on the implant's surface creating magnetic forces responsible for pulling MNP-loaded cells. Although this model system does not accurately simulate all *in vivo* conditions, it subjects the MNP-loaded ECs to some of the conditions that would be experienced *in vivo*, such as flow-derived shear force and physical force due to capture and adhesion of cells onto a metal surface. To understand the influence of each particular effect (e.g., MNPs, flow-derived shear force and cell capture and adhesion) on gene expression of MNP-loaded and mesh captured cells, we normalized gene expression of MNP-loaded and captured cells to three different controls. The control groups include: nonloaded circulated RAECs exposed to a magnetic field, isolating effects of MNPs and cell capture; MNP-loaded RAECs at static condition, isolating effects of flow and cell capture; and MNP-loaded RAECs circulated w/o exposure to a magnetic field, isolating the effect of cell capture. As expected, no cells were captured on the mesh when we ran nonloaded RAECs or MNP-loaded RAECs without exposure to a magnetic field. However, efficient cell capture was observed for MNP-loaded RAECs under magnetic conditions as evidenced by fluorescence of MNPs within the cells (in red) or by fluorescence of live cells (in green) stained with the CellTrace™ Calcein Green AM (Life Technology, USA) (Figure 5A). Mesh captured MNP-loaded RAECs were incubated for 24 h to evaluate their proliferation potential and ability to endothelialize the stainless steel surface. Captured cells adhered, spread and covered the mesh surface over 24 h, demonstrating that biological interaction with stainless steel is possible when used *in vivo* (Figure 5B).

The Rat Endothelial Cell Biology RT² Profiler™ PCR Array-based gene-expression profile of MNP-loaded RAECs captured on mesh surface against each of the three controls was performed and the genes that showed more than twofold regulation with $p < 0.05$ are listed in the Table 2. Similar to our study of gene expression of MNP-loaded RAECs at static conditions, most of the 84 genes remained unchanged for the mesh captured cells during 1 h of exposure to a magnetic field. Gene expression of captured cells normalized to Control 1 (nonloaded circulating cells, isolating effects of MNPs and cell capture) did not show any significant ($p < 0.05$) changes. Interestingly, most of the genes that showed expression changes at the static conditions (purely due to MNP loading, Table 1) remained unchanged except the genes involved in inflammatory responses. Control 1 per Table 2 was designed to observe the effects of both MNPs and cell capture onto a stainless steel mesh surface and we expected to observe similar changes in gene expression as observed due to MNP loading at static conditions (per Table 1). However, none of the studied 84 genes in cell capture experiment normalized to Control 1 (nonloaded circulated cells) showed significant change in expression.

Normalization of MNP-loaded and mesh captured cells to Controls 2 and 3 represents genes, which are possibly affected by flow and cell capture. The common inflammatory genes such as *Ccl2*, *Cxcl1* and *IL6* were upregulated (Control 3 per Table 2). It is clear that some inflammatory genes displayed in the Table 2 are quite vulnerable to multiple types of stress involving flow-derived shear force, physical capture and adhesion onto a metal surface. It is also evident that the only gene affected by cell capture on metal surface is *Ptgs2* (Control 2 per Table 2), as its expression did not show significant changes at static conditions.

Discussion

Our group investigates whether magnetically targeted ECs to intravascular stents will enable restoration of injured endothelium and prevent the recurrence ISR in a rat carotid stent angioplasty artery model. In this methodology, the therapeutic agents, MNP-loaded ECs, are targeted to the surface of a stent magnetized by a homogeneous magnetic field [10]. MNP-loading and physical targeting may potentially affect metabolic integrity of delivered cells compromising their normal function. Therefore, it is important to assess cells' metabolic status and functional potency. Thus, the goal of the present study was to determine to what extent MNP-loading modulates EC function and gene-expression profile. Our study reports three important findings. First, using *in vitro* endothelial functional assays, we have demonstrated that endothelial tube formation activity, adhesion to various biological substrates and NO production were normal in MNP-loaded RAECs compared with unloaded cells. Second, compared with unloaded cells, MNP-loaded RAECs revealed modulated transcript levels of several endothelial genes intimately involved in angiogenesis, endothelial and vascular SMC (VSMC) growth, proliferation and survival. These changes in the transcript levels of endothelial genes in MNP-loaded RAECs were not observed when cells were captured onto the mesh surface. Third, we have also noted significant attenuation of transcript levels of genes involved in blood coagulation. Collectively, our findings identify that MNP-loading in RAECs does not adversely affect functional nature of cells, inducing expression of genes involved in EC growth and survival, while repressing expression of genes involved in coagulation.

Neointimal hyperplasia is a major contributing factor for ISR [4]. Although the precise molecular and cellular mechanism underlying ISR is still being corroborated, the general consensus is that the thrombotic environment triggered by activation of platelets, macrophages and leukocytes as well as induction of SMC proliferation, migration, and deposition of ECM primarily contribute to ISR [5]. In addition to these cellular processes, additional risk factors of restenosis include diabetes, residual stenosis, number of stents and the stent length. It has been postulated that reendothelialization and repression of thrombosis would be critical for the long-term outcome of stent implantation. Consistent with this concept, anticoagulant and antiplatelet therapy reduced thrombosis and delayed the development of restenosis [23]. It is worth noting that compared with unloaded RAECs, transcript levels of selective endothelial genes involved in angiogenesis and endothelial homeostasis were elevated or remain unchanged, while transcript levels of genes involved in vasodilatation and thrombosis were attenuated in MNP-loaded RAECs (Table 1).

NO released by ECs governs EC proliferation, homeostasis and angiogenesis. NO possess a number of antiatherogenic properties, including inhibition of platelet and monocyte adhesion [24,25], as well as SMC proliferation and migration [26,27]. In addition, NO is also a potent vasodilator acting to maintain vascular tone and function within the vessel wall. Therefore, elevated transcript levels of *Nos3* (also known as *eNos*) in MNP-loaded RAECs holds the promise of attenuating neointimal hyperplasia following stent implantation (Table 1). Quantification of total cumulative NO produced by MNP-loaded and unloaded cells was found to be comparable per Figure 4B. This result indicates that MNPs have no adverse effects on generation of NO and that MNP-loaded cells are expected to function as unloaded cells after targeting to stents.

In contrast to *Nos3*, we noted significant attenuation of transcript levels of *Edn1* (Table 1), which encodes Et-1, an endothelium-derived contracting peptide with potent vasoconstriction effects [28]. Binding of Et-1 activates its receptors, endothelin type A (ETA) and type B (ETB), to trigger its physiologic action on vasoconstriction and proliferation of VSMC [29]. In addition, activation of ETB also stimulates release of vasorelaxant agents such as NO and prostacyclin (PGI₂) [30]. A previously reported study demonstrated positive correlation between the severity of atherosclerosis and the concentration of circulating Et-1, implicating a molecular link between Et-1 activity and pathophysiology of CVD [31]. Indeed, Et-1 antagonists have been shown to decrease the size of atheroma in humans [32]. Based on this literature data, we hypothesize that significant attenuation of *Edn1* transcript levels in MNP-loaded RAECs may contribute to a lower vasoconstrictor response following stent implantation, and then lead to a lower incidence of ISR.

Although a modest reduction of transcript levels of *VEGF receptor 1* (*VEGFR1/Flt-1*) gene was observed in MNP-loaded RAECs (per Table 1), the transcript levels of *VEGFR2* (also known as *Flk1*) and their common ligand, VEGF were similar in MNP-loaded and unloaded RAECs. Similarly, the expression level of *Tie2* (also known as *Tek*) involved in angiogenesis remained unchanged. These data collectively support our hypothesis that MNP loading does not adversely affect endothelial gene program and cell function *in vitro*. Consistent with this notion, NO production, adhesion and endothelial tube formation were

shown to be normal in both MNP-loaded and unloaded RAECs (Figures 2C & 3A). These results correlate well with our previous findings demonstrating that in both two- and three-dimensional migration studies cell motility was not impaired despite the inclusion of MNPs at the similar level of loading [17]. These results are also in line with previously reported observations that superparamagnetic iron oxide nanoparticles had no effects on important cell functions [33,34]. Intriguingly, modulation of transcript levels of endothelial genes in MNP-loaded RAECs did not persist when those cells were captured on mesh in a flow system (Table 2). Transcript level of some inflammatory genes (Table 2) showed elevation in mesh captured RAEC, implicating that flow-derived shear force or physical force generated due to cell capture may result in the induction of inflammatory response genes. Nonetheless, further studies are required to clarify the reason behind inflammatory response gene's upregulation.

We noted a modest reduction in the transcript level of *F3* gene encoding coagulation factor III, Tf, which is also known as CD142. It acts as the high-affinity receptor for the coagulation factor VII and forms a complex which provides a catalytic event responsible for initiation of the coagulation protease cascades by specific limited proteolysis [35]. Considering the fact that repression of thrombosis is critical for the long-term outcome of stent implantation, we anticipate that down regulation of thromboplastin may contribute to an anti-coagulatory environment for preventing restenosis. However, the downregulation of thromboplastin may be restored back to a baseline level after cell proliferation on the stent and subsequent dilution or loss of intracellular MNPs. We observed that after six cell divisions the MNPs were not detectable within cells.

Similarly, we found significant reduction in transcript levels of two other prothrombotic genes, *Ser-pine1* and *Thbs1*. *Ser-pine1* encodes Pai-1, a member of the Serpin (serine protease inhibitor) superfamily, the major physiological inhibitor of t-Pa and urokinase uPa *in vivo*, which plays a critical role in regulation of fibrinolysis [36]. As t-Pa is very important in anti-coagulatory response, we hypothesize that significant reduction of *Ser-pine1* gene transcript may help maintain antithrombotic environment and thus, lessen the possibility of initial trigger of intimal hyperplasia. *Thbs1* gene encodes the matrix protein Tsp1, which is a 450-kDa homotrimeric glycoprotein that interacts with multiple extracellular macromolecules and cell surface receptors, thus exerting a wide range of functions [37,38]. The spontaneous development of angiogenic tube-like structures is also inhibited by Tsp1 both *in vitro* and *in vivo* [39]. In marked contrast, Tsp1 promotes VSMC proliferation and migration [40,41] and plays a stimulatory role in platelet activation and aggregation [42,43]. Correlating with the report by Donghui Chen *et al.* on expedited reendothelialization and reduced neointimal thickening due to delivery of the monoclonal neutralizing anti-Tsp1 antibody locally to the arterial wall after balloon angioplasty in the rat carotid artery [44], it is quite reasonable to assume that a decreased level of *Thbs1* gene transcript could be very promising to enhance re-endothelialization and reduce neointimal thickening in the stented blood vessel. However, all of the gene expression-based hypotheses could not be interpreted as evidence of efficacy without further validation. Future *in vivo* studies will be required to confirm whether observed *in vitro* functional

potency and gene expression would lead to a therapeutic efficacy in treating of ISR using magnetic cell delivery strategy.

Currently, the molecular mechanism underlying MNP-dependent differential modulation of gene expression in RAECs is unknown. Therefore, future studies are needed to decipher whether MNP loading activates and represses certain signaling pathways, which contribute to induction or repression of transcription of respective downstream target genes. In addition, we cannot rule out the possibility that MNP loading might positively and negatively modulate the half-life of gene transcription. It is noteworthy, that alternation of gene transcript levels in MNP-loaded RAECs at static conditions was reversed when MNP-loaded RAECs were captured onto a stainless steel mesh (compare Table 1 & 2). For example, decrease of *Thbs1* transcript levels in MNP-loaded RAECs (Table 1) was not observed when cells were captured onto mesh (Table 2). Although the molecular mechanism by which targeting and attaching to mesh surface modifies the effects of MNPs is not completely understood, it is conceivable that loading and targeting to the mesh exhibit opposite action on *Thbs1* expression. Indeed, Pallero MA et al. have reported that stainless steel ions stimulate *Thbs1* expression through attenuating PKG activity in a catalase-dependent mechanism [45]. Therefore, it is plausible that MNPs reduce *Thbs1* expression by activating PKG activity. Future study will be required to test whether similar mechanistic phenomenon exists for the elevation and reduction of transcript levels of other genes.

Conclusion

We have described in depth characterization of gene expression and functional behavior of MNP-loaded and implant-targeted primary ECs. Importantly, we have observed upregulation of certain genes involved in angiogenesis, atherosclerosis and inflammation. Our results are specific to the MNP type used in this study and could not be directly extrapolated to other types of magnetic particles. Collectively, our data suggest that targeting MNP-loaded ECs to vascular stents may potentially lead to beneficial effects by stimulating angiogenesis leading to re-endothelialization of an implant and attenuating neointimal hyperplasia. Therefore, magnetic EC targeting strategy in combination with the currently available pharmacological and surgical approaches for cardiovascular treatment may potentially provide novel therapeutic direction with clinical benefits to attenuate recurrence of ISR.

Future perspective

Magnetically mediated delivery of ECs to vascular stents is an attractive and unique cell-based therapeutic strategy that may potentially minimize recurrence of ISR. However, for successful translation of this approach for humans a number of challenges still have to be overcome.

The MNPs need to be further developed to retain the most efficient balance in their magnetic properties and biocompatibility. Because MNPs in particular and other types of nanoscale carriers in general may interfere with cellular function on various levels, there is a growing need to systematically evaluate the potential adverse effects of the nanoscale carriers on

cellular function and normal cell physiology. Future clinical implementation of nanoscale carriers will require establishment of international toxicity and biocompatibility standards for these systems. Such standardized assessments will provide a comprehensive picture on biocompatibility and therapeutic potential status of cell-based therapy and enable their safe clinical use.

It is very possible that future cell-based therapeutic strategies will be centered on use of specialized autologous cells that will be obtained either by controlled differentiation or reprogramming from patient's own stem or other cell types. In this regard, the future development of magnetic cell targeting will involve improvement of MNP loading efficiency in 'hard-to-load' cells. Such strategies, for example, may include application of magnetic pulling forces in combination with oscillating magnetic fields as has been shown to enhance transfection in 'hard-to-transfect' human airway epithelial [46] and neural cells [47,48].

Use of magnetizable implants (in particular intravascular stents) in combination with a modest uniform magnetic field for generation of both high field gradients and sufficiently strong magnetizing field at the target site seems to be scalable to human dimensions and therefore has a great potential to be translated into clinical use. To this end, the chemical composition of implant material and its geometry will play a key role in balancing the best magnetic properties for achieving efficient magnetic cell targeting with implant's biocompatibility. Today, stents are usually composed of the medical-grade stainless-steel alloy 316L, which exhibits a minimal response to external magnetic fields. In our magnetic cell targeting approach we use a more magnetically responsive alloy, such as a 304-grade stainless steel, due to its balance of sufficient magnetic properties and its corrosion resistance in aqueous environment. While this material performs well in short-term magnetic cell targeting experiments, evaluation of its long-term corrosion resistance and biocompatibility as well as its approval by the regulatory agencies will be mandatory to enable its clinical use.

Finally, future preclinical studies have to focus on the therapeutic outcomes of the magnetic cell therapy in attenuating target pathological conditions. For example, in treatment of ISR, delivery of cells displaying endothelial phenotype (with or without genetically enhanced endothelial function) to stented blood vessels is expected to minimize neointimal hyperplasia through reconstitution of injured endothelium in the stented blood vessel. It is also clear that successful translation of magnetic-cell-based therapy to the clinic will require the joint efforts of researchers from multiple disciplinary backgrounds in order to overcome the above challenges.

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References

Papers of special note have been highlighted as:

- of interest;

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•• of considerable interest

1. Writing Group M, Lloyd-Jones D, Adams RJ, et al. Heart disease and stroke statistics – 2010 update: a report from the American Heart Association. *Circulation*. 2010; 121(7):e46–e215. [PubMed: 20019324]
2. Roger VL, Go AS, Lloyd-Jones DM, et al. Heart disease and stroke statistics – 2012 update: a report from the American Heart Association. *Circulation*. 2012; 125(1):e2–e220. [PubMed: 22179539]
3. Flammer AJ, Anderson T, Celermajer DS, et al. The assessment of endothelial function: from research into clinical practice. *Circulation*. 2012; 126(6):753–767. [PubMed: 22869857]
4. Pauletto P, Sartore S, Pessina AC. Smooth-muscle-cell proliferation and differentiation in neointima formation and vascular restenosis. *Clin. Sci.* 1994; 87(5):467–479. [PubMed: 7874832]
- 5••. Scott NA. Restenosis following implantation of bare metal coronary stents: pathophysiology and pathways involved in the vascular response to injury. *Adv. Drug Deliv. Rev.* 2006; 58(3):358–376. [PubMed: 16733073] Excellent review that summarizes the restenotic process that occurs after the implantation of bare metal coronary stents.
- 6••. Er OB, Ma X, Simard T, Pourdjabbar A, Hibbert B. Pathogenesis of neointima formation following vascular injury. *Cardiovasc. Hematol. Disord. Drug Targets*. 2011; 11(1):30–39. [PubMed: 21446903] Great review discussing key studies of neointima development following vascular injury with a focus on the origin of cells participating in neointima formation.
7. Asahara T, Chen D, Tsurumi Y, et al. Accelerated restitution of endothelial integrity and endothelium-dependent function after phVEGF165 gene transfer. *Circulation*. 1996; 94(12):3291–3302. [PubMed: 8989143]
8. Hedman M, Hartikainen J, Syvanne M, et al. Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). *Circulation*. 2003; 107(21):2677–2683. [PubMed: 12742981]
9. Losordo DW, Isner JM, Diaz-Sandoval LJ. Endothelial recovery: the next target in restenosis prevention. *Circulation*. 2003; 107(21):2635–2637. [PubMed: 12782613]
- 10••. Polyak B, Fishbein I, Chorny M, et al. High field gradient targeting of magnetic nanoparticle-loaded endothelial cells to the surfaces of steel stents. *Proc. Natl Acad. Sci. USA*. 2008; 105(2):698–703. [PubMed: 18182491] High-quality study demonstrating the feasibility of magnetically-mediated endothelial cell targeting to magnetizable intravascular stents *in vitro* and *in vivo*.
11. Finn AV, Gold HK, Tang A, et al. A novel rat model of carotid artery stenting for the understanding of restenosis in metabolic diseases. *J. Vasc. Res.* 2002; 39(5):414–425. [PubMed: 12297704]
12. Fishbein I, Alferiev I, Bakay M, et al. Local delivery of gene vectors from bare-metal stents by use of a biodegradable synthetic complex inhibits in-stent restenosis in rat carotid arteries. *Circulation*. 2008; 117(16):2096–2103. [PubMed: 18413497]
13. Voyta JC, Via DP, Butterfield CE, Zetter BR. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J. Cell Biol.* 1984; 99(6):2034–2040. [PubMed: 6501412]
14. Macdonald C, Friedman G, Alamia J, Barbee K, Polyak B. Time-varied magnetic field enhances transport of magnetic nanoparticles in viscous gel. *Nanomedicine*. 2010; 5(1):65–76. [PubMed: 20025465]
15. Johnson B, Toland B, Chokshi R, Mochalin V, Koutzaki S, Polyak B. Magnetically responsive paclitaxel-loaded biodegradable nanoparticles for treatment of vascular disease: preparation, characterization and *in vitro* evaluation of anti-proliferative potential. *Curr. Drug Deliv.* 2010; 7(4):263–273. [PubMed: 20695837]
16. Adams CF, Rai A, Sneddon G, Yiu HH, Polyak B, Chari DM. Increasing magnetite contents of polymeric magnetic particles dramatically improves labeling of neural stem cell transplant populations. *Nanomedicine*. 2014; 11(1):19–29. [PubMed: 25038496]
17. Macdonald C, Barbee K, Polyak B. Force dependent internalization of magnetic nanoparticles results in highly loaded endothelial cells for use as potential therapy delivery vectors. *Pharm. Res.* 2012; 29(5):1270–1281. [PubMed: 22234617]

18. Arnaoutova I, George J, Kleinman HK, Benton G. The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis*. 2009; 12(3):267–274. [PubMed: 19399631]
19. Arnaoutova I, Kleinman HK. *In vitro* angiogenesis: endothelial cell tube formation on gelled basement membrane extract. *Nat. Protoc*. 2010; 5(4):628–635. [PubMed: 20224563]
- 20•. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler. Thromb. Vasc. Biol*. 2003; 23(2):168–175. [PubMed: 12588755] Discusses the role of endothelial dysfunction as a potential marker of the inherent atherosclerotic risk in an individual.
21. Clowes AW, Karnowsky MJ. Suppression by heparin of smooth muscle cell proliferation in injured arteries. *Nature*. 1977; 265(5595):625–626. [PubMed: 859561]
22. Snow AD, Bolender RP, Wight TN, Clowes AW. Heparin modulates the composition of the extracellular matrix domain surrounding arterial smooth muscle cells. *Am. J. Pathol*. 1990; 137(2):313–330. [PubMed: 2386199]
23. Leon MB, Baim DS, Popma JJ, et al. A clinical trial comparing three antithrombotic-drug regimens after coronary-artery stenting. Stent Anticoagulation Restenosis Study Investigators. *N. Engl. J. Med*. 1998; 339(23):1665–1671. [PubMed: 9834303]
24. Mellion BT, Ignarro LJ, Ohlstein EH, Pontecorvo EG, Hyman AL, Kadowitz PJ. Evidence for the inhibitory role of guanosine 3', 5'-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood*. 1981; 57(5):946–955. [PubMed: 611365]
25. Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl Acad. Sci. USA*. 1991; 88(11):4651–4655. [PubMed: 1675786]
26. Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest*. 1989; 83(5):1774–1777. [PubMed: 2540223]
27. Sarkar R, Meinberg EG, Stanley JC, Gordon D, Webb RC. Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells. *Circ. Res*. 1996; 78(2):225–230. [PubMed: 8575065]
28. Yanagisawa M, Kurihara H, Kimura S, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*. 1988; 332(6163):411–415. [PubMed: 2451132]
29. Sakurai T, Yanagisawa M, Masaki T. Molecular characterization of endothelin receptors. *Trends Pharmacol. Sci*. 1992; 13(3):103–108. [PubMed: 1315462]
30. Rautureau Y, Schiffrin EL. Endothelin in hypertension: an update. *Curr. Opin. Nephrol. Hyperten*. 2012; 21(2):128–136.
31. Lerman A, Holmes DR Jr, Bell MR, Garratt KN, Nishimura RA, Burnett JC Jr. Endothelin in coronary endothelial dysfunction and early atherosclerosis in humans. *Circulation*. 1995; 92(9):2426–2431. [PubMed: 7586341]
32. Yoon MH, Reriani M, Mario G, et al. Long-term endothelin receptor antagonism attenuates coronary plaque progression in patients with early atherosclerosis. *Int. J. Cardiol*. 2013; 168(2):1316–1321. [PubMed: 23290081]
- 33•. Cheng K, Li TS, Malliaras K, Davis DR, Zhang Y, Marban E. Magnetic targeting enhances engraftment and functional benefit of iron-labeled cardiosphere-derived cells in myocardial infarction. *Circ. Res*. 2010; 106(10):1570–1581. [PubMed: 20378859] Discusses the role of endothelial dysfunction as a potential marker of the inherent atherosclerotic risk in an individual.
- 34•. Cheng K, Malliaras K, Li TS, et al. Magnetic enhancement of cell retention, engraftment, and functional benefit after intracoronary delivery of cardiac-derived stem cells in a rat model of ischemia/reperfusion. *Cell Transplant*. 2012; 21(6):1121–1135. [PubMed: 22405128] Demonstrates that magnetically enhanced intracoronary cell delivery used in a rat model of ischemia/reperfusion injury is safe and improves cell therapy outcomes.
35. Moons AH, Levi M, Peters RJ. Tissue factor and coronary artery disease. *Cardiovasc. Res*. 2002; 53(2):313–325. [PubMed: 11827681]
36. Gils A, Declerck PJ. Plasminogen activator inhibitor-1. *Curr. Med. Chem*. 2004; 11(17):2323–2334. [PubMed: 15379715]

37. Frazier WA. Thrombospondins. *Curr. Opin. Cell Biol.* 1991; 3(5):792–799. [PubMed: 1718338]
38. Asch AS, Tepler J, Silbiger S, Nachman RL. Cellular attachment to thrombospondin. Cooperative interactions between receptor systems. *J. Biol. Chem.* 1991; 266(3):1740–1745. [PubMed: 1703153]
39. Iruela-Arispe ML, Bornstein P, Sage H. Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells. *in vitro. Proc. Natl Acad. Sci. USA.* 1991; 88(11):5026–5030.
40. Majack RA, Cook SC, Bornstein P. Control of smooth muscle cell growth by components of the extracellular matrix: autocrine role for thrombospondin. *Proc. Natl Acad. Sci. USA.* 1986; 83(23):9050–9054. [PubMed: 3491367]
41. Yabkowitz R, Mansfield PJ, Ryan US, Suchard SJ. Thrombospondin mediates migration and potentiates platelet-derived growth factor-dependent migration of calf pulmonary artery smooth muscle cells. *J. Cell. Physiol.* 1993; 157(1):24–32. [PubMed: 8408239]
42. Tuszynski GP, Rothman VL, Murphy A, Siegler K, Knudsen KA. Thrombospondin promotes platelet aggregation. *Blood.* 1988; 72(1):109–115. [PubMed: 3390605]
43. Dixit VM, Haverstick DM, O'Rourke KM, et al. A monoclonal antibody against human thrombospondin inhibits platelet aggregation. *Proc. Natl Acad. Sci. USA.* 1985; 82(10):3472–3476. [PubMed: 2582413]
44. Chen D, Asahara T, Krasinski K, et al. Antibody blockade of thrombospondin accelerates reendothelialization and reduces neointima formation in balloon-injured rat carotid artery. *Circulation.* 1999; 100(8):849–854. [PubMed: 10458722]
45. Pallero MA, Talbert Roden M, Chen YF, et al. Stainless steel ions stimulate increased thrombospondin-1-dependent TGF-beta activation by vascular smooth muscle cells: implications for in-stent restenosis. *J. Vasc. Res.* 2010; 47(4):309–322. [PubMed: 20016205]
- 46••. McBain SC, Griesenbach U, Xenariou S, et al. Magnetic nanoparticles as gene delivery agents: enhanced transfection in the presence of oscillating magnet arrays. *Nanotechnology.* 2008; 19(40):405102. [PubMed: 21832609] Interesting methodology demonstrating that application of magnetic field gradients in combination with oscillating magnetic fields significantly enhances transfection efficiency.
- 47•. Jenkins SI, Pickard MR, Granger N, Chari DM. Magnetic nanoparticle-mediated gene transfer to oligodendrocyte precursor cell transplant populations is enhanced by magnetofection strategies. *ACS Nano.* 2011; 5(8):6527–6538. [PubMed: 21721568] Study that tested the feasibility of using physical delivery methods, employing static and oscillating field 'magnetofection' techniques to enhance magnetic nanoparticle-mediated gene transfer in 'hard-to-transfect' neural cells.
48. Pickard M, Chari D. Enhancement of magnetic nanoparticle-mediated gene transfer to astrocytes by 'magnetofection': effects of static and oscillating fields. *Nanomedicine.* 2010; 5(2):217–232. [PubMed: 20148634]

Executive summary

Isolation & characterization of primary endothelial cells

- Highly pure culture of primary rat aortic endothelial cells (RAECs) was isolated and characterized.
- Primary endothelial cells (ECs) were capable to internalize approximately 25 pg magnetite per cell, which conferred them sufficient magnetic responsiveness and enabled their efficient targeting to a magnetizable implant.

Functional behavior of magnetic nanoparticle-loaded primary ECs

- Loading primary ECs with polymeric magnetic nanoparticles (MNPs) does not adversely affect their functional behavior as was evidenced by normal endothelial tube formation, adhesion to various biological substrates and production of nitric oxide assays.
- Magnetically captured RAECs adhered, spread and covered mesh surface over 24 h, demonstrating that biological interaction with stainless steel is possible and can be achieved *in vivo*.

Gene-expression profile of MNP-loaded RAECs at static conditions and targeted *in vitro*

- In static conditions, compared with unloaded cells, MNP-loaded RAECs revealed modulated transcript levels of several endothelial genes intimately involved in angiogenesis, endothelial and vascular smooth muscle cell growth, proliferation and survival. MNP loading also resulted in significant attenuation of transcript levels of genes involved in blood coagulation.
- The changes in transcript levels of endothelial genes at static conditions in MNP-loaded RAECs were not observed when cells were captured onto a metal mesh surface. Only few genes involved in inflammatory response were upregulated after cell capture to a magnetizable implant.

Conclusion

- Collectively, our findings show that MNP-loading in RAEC does not adversely affect the nature of cells, inducing expression of genes involved in EC growth and survival, while repressing expression of genes involved in coagulation.
- Gene expression and functional behavior indicate that targeting MNP-loaded ECs to vascular stents may potentially introduce beneficial effects by stimulating re-endothelialization of an implant and attenuating neointimal hyperplasia.
- Our results are specific to the MNP type used in this study and could not be directly extrapolated to other types of magnetic particles.

Figure 1. Characterization of primary rat aortic endothelial cells

(A) Cobblestone morphology of rat aortic endothelial cells by phase contrast microscopy.

(B) Uptake of acetylated low-density lipoprotein (LDL): acetylated LDL: green; nuclei: red.

Immunostaining by fluorescence microscopy. (C) VE-Cad (marker: green; nuclei: red). (D)

CD31 (marker: red; nuclei: green). (E) Tie-2 (marker: red; nuclei: green). (F) α-SMA

(marker: red; nuclei: green). Immunostaining by flow cytometry: purple colored histograms

represent cells stained with antibodies specific for the indicated antigens (G) CD31, (H)

Tie-2, (I) α-SMA and blue histograms represent background fluorescence obtained by

staining the same cells with isotype-matched control antibodies. These results demonstrate

that 98% of the cells are CD31 (endothelial cell-specific marker) positive, 90% Tie-2

positive and only 4% of the cells are α-SMA (smooth muscle cell marker) positive.

For color figures, please see online at [www.futuremedicine.com/doi/full/10.2217/NNM.](http://www.futuremedicine.com/doi/full/10.2217/NNM.15.13)

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Figure 2. Magnetic nanoparticle-loading studies

(A) Flow cytometry analysis show that 96% of the RAECs are loaded with MNPs. Based on the spectrophotometric determination of iron, the magnetite content was estimated to be 25.3 ± 0.75 pg magnetite/cell. (B) Fluorescent and phase contrast images of RAECs loaded with MNPs. MNPs are covalently labeled with 564/570 nm BODIPY[®] fluorophore.

FSC: Forward-scattered light, proportional to cell-surface area or size; MNP: Magnetic nanoparticle; RAEC: Rat aortic endothelial cell; SSC: Side-scattered light, proportional to cell granularity or internal complexity.

Figure 3. *In vitro* tube formation assay

(A) Phase contrast and fluorescent images of the tube formation on Matrigel™ by primary nonloaded and MNP-loaded RAECs (25 pg magnetite/cell) at different time points. (B) Quantitative comparison of the tube formation of nonloaded and MNP-loaded RAECs at 4 and 12 h based on four parameters: number of tubes, loops, branching points and covered area as estimated by Wimasis image analysis software (n = 4; *p < 0.05).

MNP: Magnetic nanoparticle; RAEC: Rat aortic endothelial cell.

Figure 4. Adhesion and nitric oxide production assay

(A) Adhesion assay of MNP-loaded rat aortic endothelial cells compared with nonloaded rat aortic endothelial cells on different matrices ($n = 2$). (B) Cumulative NO production profile of unloaded and MNP-loaded cells at two MNP doses (25 and 12.5 μg magnetite/cell). The NO amounts are normalized to the total protein content in each cell sample to compare NO levels per cell.

MNP: Magnetic nanoparticle; NO: Nitric oxide.

Figure 5. *In vitro* targeting of the magnetic nanoparticle-loaded rat aortic endothelial cells onto a stainless steel mesh under flow conditions

(A) Magnetically responsive rat aortic endothelial cells are captured on mesh during 1-h exposure to a magnetic field as evidenced by the red fluorescence of magnetic nanoparticles and CellTrace™ Calcein Green AM staining of the live cells. (B) After capture on mesh, cells attach, spread and efficiently endothelialize the surface of the bare metal mesh over 24 h.

Table 1
Genes showed up or downregulation in magnetic nanoparticle-loaded rat aortic endothelial cells at static conditions versus nonloaded rat aortic endothelial cells (n = 3)

Gene symbol	Gene bank identifier	Protein symbol	Function category	Fold regulation	p-value
<i>Agt</i>	NM_134432	Agt	Inflammatory response, vasoconstriction and vasodilation	2.38	0.007
<i>Cxcr5</i>	NM_053303	Cxcr5	Inflammatory response	2.98	0.050
<i>Edn1</i>	NM_012548	Edn1	Angiogenesis, vasoconstriction and vasodilation	-4.25	0.030
<i>F3</i>	NM_013057	Tf	Coagulation and angiogenesis	-2.65	0.008
<i>Flt1</i>	NM_019306	Flt1	Angiogenesis	-3.10	0.0001
<i>Nos3</i>	NM_021838	Nos3 (eNos)	Angiogenesis	2.19	0.036
<i>Serpine1</i>	NM_012620	Pai-1 (Serpin E1)	Cell adhesion and platelet activation	-6.16	0.0003
<i>Thbs1</i>	NM_001013062	Thbs1	Cell adhesion, coagulation, platelet activation and apoptosis	-4.38	0.000006

Table 2
Genes altered in mesh captured magnetic nanoparticle-loaded rat aortic endothelial cells versus various control conditions

Gene symbol	Gene bank	Protein symbol	Function category	Fold regulation	p-value
Control 1: Comparison to nonloaded circulated RAECs (n = 3). Effects of MNPs and cell capture					
There were no statistically significant changes in gene alterations with $p < 0.05$					
Control 2: Comparison to MNP-loaded RAEC at static conditions (n = 3). Effects of flow and cell capture					
<i>Ptgs2</i>	NM_01722	Ptgs2	Inflammatory response, angiogenesis, vasoconstriction and vasodilation	4.30	0.02
Control 3: Comparison to MNP-loaded circulated RAECs without exposure to a magnetic field (n = 3). Effect of cell capture					
<i>Ccl2</i>	NM_031530	Ccl2	Inflammatory response, angiogenesis and apoptosis	2.72	0.050
<i>Cxcl1</i>	NM_030845	Cxcl1	Inflammatory response	2.40	0.008
<i>IL6</i>	NM_012589	IL-6	Inflammatory response, platelet activation and angiogenesis	3.69	0.050

MNP: Magnetic nanoparticle; RAEC: Rat aortic endothelial cell.