There is increasing interest in developing bioconjugated carriers for the cellular delivery of bioactive molecules to stem cells, since they can allow modulation of stem cell differentiation. The present study reported biocompatible silica nanoparticle–insulin conjugates for rat mesenchymal stem cell (RMSC) adipogenic differentiation in vitro. A systematic study was first carried out on the biocompatibility of the SiNPs with RMSCs. The cell viability assay was performed to screen the SiNP concentration for creating little cytotoxicity on RMSCs. Furthermore, transmission electron microscopy (TEM) and adipogenesis and osteogenesis assays revealed that the pure SiNPs had no effect on cellular ultrastructures, adipogenic differentiation, and osteogenic differentiation. Under the optimized SiNP concentration with little cytotoxicity on RMSC and no effects on the RMSC phenotype, SiNP–insulin conjugates were prepared and used for RMSC adipogenic differentiation. Results showed that RMSCs had the ability to differentiate into adipocytes when cultured in the presence of insulin-conjugated SiNPs. This work demonstrated that the biological activity of insulin conjugated to the SiNPs was not affected and the SiNPs could be used as biocompatible carriers of insulin for RMSC adipogenic differentiation, which would help to expand the new potential application of SiNPs in stem cell research.

INTRODUCTION

Ongoing advances in the directed differentiation in vitro of stem cells have offered great prospects for medical research and opened new avenues for therapy in many human disorders (1–3). Until now, various strategies have been developed and employed to direct and control stem cell differentiation in vitro, including development of defined culture milieu (4), use of extracellular matrix (5, 6), co-culturing with fully differentiated somatic cell types (7, 8), up-regulating the expression of connexin proteins to enhance the gap junction formation and intercellular coupling (9), and directing stem cell differentiation through genetic modulation (10). Among the strategies to achieve controlled differentiation of stem cells into well-defined lineages in vitro, bioactive molecules such as cytokines, growth factors, and small chemicals as defined culture conditions have been widely used to promote stem cell differentiation in vitro (11). Especially for the adult stem cells, differentiation cannot appear spontaneously in the absence of cytokines and growth factors.

For stem cell differentiation in vitro using bioactive molecules as defined culture conditions, the differentiation efficiency would usually be affected not only by the internal factors such as genetic, developmental potential of stem cells, but also by the characteristics of exogenous bioactive molecules, such as their biological activity (12), solubility (13), and cell toxicity (14).

Although endogenous bioactive molecules could be produced with recombinant DNA constructs through genetic manipulation and have successfully induced the transfected stem cell differentiation in vitro, the risk of mutagenesis and/or oncogenesis of recombinant DNA and the viral-based vectors is still unpredictable (15). Therefore, the development of novel strategies to deliver exogenous bioactive molecules to the stem cells is still regarded as a hot issue for stem cell differentiation in vitro.

Recently, the wide spectrum of nanotechnologies holds great promise for the study of stem cell biology and the development of new approaches to stem cell research (16). The potential applications of nanotechnologies in stem cell research include magnetic nanoparticles for stem cell isolation and sorting (17), the fluorescent nanoparticle labeling method for stem cell labeling and stem cell in vivo tracking (18), engineering nanometer-scale materials for proliferation and differentiation of stem cells (19, 20), and nanomaterial-based carriers for the intra/extracellular delivery of genes, drugs, and bioactive molecules for stem cell differentiation (21–23). For intra/extracellular delivery of bioactive molecules to stem cells, the use of nanomaterial-based carriers is expected to maintain the biological activity and stability of bioactive molecules and likely extend the duration of exposure of cells to the bioactive molecules (24). In the case of nanomaterial-based carriers for bioactive molecules to stem cells, most efforts are focused on the development of polymeric nanoparticles. For example, a novel dexamethasone-loaded carboxymethylchitosan/poly(allylamine) dendrimer (Dex-loaded CMCht/PAMAM) nanoparticle has been designed and combined with either ceramic or polymeric scaffolds to induce the differentiation of rat bone marrow stromal cells in vitro, and it has demonstrated that Dex-loaded CMCht/PAMAM dendrimer nanoparticles could be used as intracellular nanocarriers of biological agents to modulate the early osteogenic differentiation of rat bone marrow stromal cells in vitro (22). The biocompatible and biodegradable...
poly(lactide-co-glycolide) (PLGA) particles with different sizes have also been used as a model system to design growth-factor-releasing systems for controlling the differentiation of human embryonic stem cells (hESCs) into the vascular lineage (23).

By comparing with polymer nanoparticles, silica nanoparticles (SiNPs) are of interest for their biocompatibility and their mechanical properties. Because of their unique characteristics, SiNPs have been widely studied in a range of areas including chemistry, engineering, and biomedicine, especially the application of functionalized SiNPs for cancer diagnostics and therapy (25, 26). Recently, they are also emerging as an idea agent for efficient stem cell labeling and tracking (27, 28). To our knowledge, SiNPs have not previously been used as carriers of bioactive molecules for stem cell differentiation in vitro. In the present study, the SiNP-based carriers of insulin for Sprague–Dawley (SD) rat mesenchymal stem cell (RMSC) adipogenic differentiation have been reported. The cell viability assay of RMSCs in the presence of pure SiNPs was first performed using the MTT assay and morphology test to screen the SiNP concentration needed to create little cytotoxicity in the RMSCs. After further confirmation that the pure SiNPs in the concentration needed to create little cytotoxicity had no effect on cellular ultrastructure, adipogenic differentiation, and osteogenic differentiation, the SiNPs were then selected as carriers to be conjugated with insulin, one of the important growth factor supplements for RMSC adipogenic differentiation. In vitro cell studies were carried out in order to evaluate the adipogenic efficacy of adipogenic induction media containing SiNP–insulin conjugates in the control experiments. The results showed that the biological activity of insulin conjugated to the SiNPs was not affected and the SiNP–insulin conjugates could be used for RMSC adipogenic differentiation, which would help to expand the new potential application of SiNPs in stem cell research.

**EXPERIMENTAL PROCEDURES**

**Materials.** Tris(2,2-bipyridyl)dichlororuthenium(II) hexahydrate (Rupy), Triton X-100, and aspartic acid were purchased from Sigma-Aldrich. Insulin was supplied by Invitrogen. N-(Maleimidophenyl)isocyanate (PmPi) was obtained from Ther- no. 3-(4,5-Dimethyliazol-2-yl)-2,5-diphenyl tetrazolium bro- mide (MTT) was supplied by Amresco. Oil red-O and Alizarin red were purchased from Dingguo Biotech. Sprague–Dawley rat mesenchymal stem cells (SD RMSCs), trypsin–EDTA, and osteogenic buffered saline (PBS, pH 7.2–7.4) were purchased from Cyagen biosciences. LG-DMEM growth medium consisting of low-glucose Dulbecco’s modified Eagle’s medium (LG-DMEM), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 2 mM glutamine; adipogenic induction media containing LG-DMEM growth medium and other supplements (5 × 10⁻⁴ M 3-isobutyl-1-methyl-xanthine, 1 × 10⁻⁴ M indomethacin, 1 × 10⁻⁶ M dexamethasone, 0.01 mg/mL insulin); adipogenic maintenance media consisting of LG-DMEM growth medium and other supplements (0.8 mM ascorbate, 0.01 M β-glycerophosphate, 1 × 10⁻⁷ M dexamethasone) were also supplied by Cyagen Biosciences. Other chemicals if not specified were all commercially available and used as received. All aqueous solutions were prepared exclusively with 18 MΩ deionized water (Barnstead Thermolyne Nanopure, Garner, NC).

**Preparation of SiNPs and FSiNPs.** Silica nanoparticles (SiNPs) and Rupy dye-doped silica nanoparticles (FSiNPs) were synthesized using the water-in-oil (W/O) microemulsion method as reported before (29). Briefly, a solution containing cyclohexane, Triton X-100, and n-hexanol (with volume ratio 4:1:1) was mixed with adequate water and stirred for 5 min (for FSiNPs, followed by the addition of Rupy dye solutions as the core material). In the presence of tetraethylorthosilicate (TEOS), polymerization reaction was initiated by adding concentrated NH₄OH. The reaction was allowed to continue for 24 h. Then, the nanoparticles were isolated by acetone and washed with ethanol and water several times to remove surfactant molecules.

**Culture of RMSCs in Vitro.** RMSCs were seeded at a density of 4 × 10³ cells/cm² in the T25 or T75 flasks and cultured in the LG-DMEM growth medium, containing 10% FBS, 100 mg/mL streptomycin, 100 U/mL penicillin, and 2 mM glutamine. Cultures were maintained at 37 °C in a humidified incubator with 5% CO₂. The cells were then dissociated with trypsin–EDTA solution and subcultured at a 1:3 ratio when they were grown to 80%–90% confluence.

**Cell Viability and Proliferation Assay.** The effect of pure SiNP concentration on RMSC viability and proliferation was first examined using MTT reduction and morphology testing. For cell viability and proliferation experiment using MTT assay, the RMSCs were grown in 96-well plates at an initial density of 4 × 10⁴ cells/well and cultured in 200 µL LG-DMEM growth medium for 24 h. Then, the cells were exposed to the pure SiNPs with final concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1, and 2 mg/mL, respectively. After the cells were cultured in the media containing pure SiNPs with different concentrations for a further 24, 48, and 72 h, 20 µL of MTT (5 mg/mL in PBS) was added to each well and incubated with the cells at 37 °C for 4 h. Thereafter, the medium was removed and 150 µL of dimethyl sulfoxide (DMSO) was added and incubated for 10 min. After all crystals were dissolved, the optical density of each well was measured at 570 nm using a microplate reader (Bio-Rad ELISA reader, Hercules, CA). The RMSCs untreated with SiNPs were provided as the normal control group, and the LG-DMEM growth media in wells were prepared as the blank control group. The cell viability was calculated according to the following Formula 1:

\[
\text{Cell viability} \% = \left( \frac{A_{\text{SiNPs}} - A_{\text{blank control}}}{A_{\text{normal control}} - A_{\text{blank control}}} \right) \times 100\%
\]

In addition, the effects of pure SiNP concentration on the morphology of RMSCs were also investigated. Briefly, the cells were cultured in a monolayer for 24 h and fed with LG-DMEM growth medium containing pure SiNPs with different final concentrations as mentioned above for a further 72 h. Then, the media was removed, and the cells were washed twice with PBS and observed under the microscope.

**Determination of Cellular Uptake of Pure SiNPs by RMSCs and the Effect of SiNPs on Cellular Ultrastructure.** To determine the uptake of SiNPs by RMSCs, the well-grown cells were incubated with 0.1 mg/mL of Rupy-doped SiNPs (FSiNPs) suspension in LG-DMEM growth medium at 37 °C for 24, 48, and 72 h. Treated cells were then washed three times with cold PBS to remove excess FSiNPs. Then, the cells were resuspended with PBS and further stained using DAPI as a nuclear counter-stain. The fluorescent dye incorporated in FSiNPs served as a marker to determine their cellular uptake in the present study, the SiNP-based carriers of insulin for Sprague–Dawley rat mesenchymal stem cells (SD RMSCs), trypsin–EDTA, and osteogenic buffered saline (PBS, pH 7.2–7.4) were purchased from Cyagen biosciences. LG-DMEM growth medium consisting of low-glucose Dulbecco’s modified Eagle’s medium (LG-DMEM), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 2 mM glutamine; adipogenic induction media containing LG-DMEM growth medium and other supplements (5 × 10⁻⁴ M 3-isobutyl-1-methyl-xanthine, 1 × 10⁻⁴ M indomethacin, 1 × 10⁻⁶ M dexamethasone, 0.01 mg/mL insulin); adipogenic maintenance media consisting of LG-DMEM growth medium and other supplements (0.8 mM ascorbate, 0.01 M β-glycerophosphate, 1 × 10⁻⁷ M dexamethasone) were also supplied by Cyagen Biosciences. Other chemicals if not specified were all commercially available and used as received. All aqueous solutions were prepared exclusively with 18 MΩ deionized water (Barnstead Thermolyne Nanopure, Garner, NC).

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ultramicrotome. The prepared ultrathin sections (70 nm) were imaged with transmission electron microscopy (JEM-1230) at an accelerating voltage of 80 kV. The RMSCs untreated with SiNPs were also prepared for the control experiments.

Determination of the Effect of Pure SiNPs on RMSC Phenotype. Mesenchymal stem cells are multipotent stem cells which can differentiate into a variety of cell types in vitro or in vivo, including osteoblasts, adipocytes, chondrocytes, myocytes, and endotheliums (30). In order to demonstrate the effect of pure SiNPs on RMSC phenotype, adipogenesis and osteogenesis assays were conducted in the presence of pure SiNPs. The effects of pure SiNPs on adipogenic differentiation were performed as follows. RMSCs were seeded onto six-well tissue culture plates in LG-DMEM growth medium at 2 × 10⁴ cells/cm². The cells grown in culture plates were then incubated with 0.1 mg/mL pure SiNPs for 24 h and maintained in LG-DMEM growth medium until post-confluence. Then, the LG-DMEM growth medium with SiNPs was replaced with LG-DMEM growth medium. The pure SiNP-incubated RMSCs were subjected to four cycles of adipogenic induction/maintenance media. Each cycle consisted of incubation with the induction media for 3 days, followed by 1 day incubation with maintenance media. After induction, oil red-O staining was performed to investigate the presence of lipid-containing vacuoles. Briefly, the induced RMSCs were washed with PBS and fixed in a PBS solution containing 4% paraformaldehyde for 30 min, then washed with PBS twice and stained with oil red-O for 20 min. To further quantify the lipid vacuole content, the oil red O was extracted using 100% isopropanol, and absorbance was measured at a wavelength of 490 nm.

To evaluate the effect of pure SiNPs on osteogenic differentiation, RMSCs were plated in six-well tissue culture plates at 3 × 10⁴ cells/cm² and exposed to 0.1 mg/mL pure SiNPs for 24 h. After the treatment, the pure SiNP incubated RMSCs were subjected to osteogenic differentiation. The osteogenic differentiation medium was replaced every three days, and induction was continued for a four-week period. After four weeks of osteogenic differentiation, RMSCs were rinsed with PBS, fixed for 30 min in 4% paraformaldehyde, and stained in Alizarin red solution for 7 min. For quantization by calcium assay, cells stained with Alizarin red were destained with 10% cetylpyridinium chloride (CPC10%) and the absorbance of destaining solution was measured at a wavelength of 562 nm. The amount of calcium was calculated according to a standard curve of Alizarin red.

Table 1. Adipogenic Differentiation Treatment of RMSCs with Different Induction Media and Different Maintenance Media

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<td>SiNP–insulin conjugates</td>
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<td>Step 2 Maintenance Media</td>
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Figure 1. Transmission electron microscopy images of SiNPs (a), FSiNPs (b), and SiNP–insulin conjugates (c).
Preparation and Characterization of SiNP–Insulin Conjugates. Under the optimized SiNP concentration with little cytotoxicity on the RMSC and no effect on the RMSC phenotype, SiNPs as biocompatible carriers of insulin for RMSC adipogenic differentiation were investigated. Insulin was conjugated to the surface of SiNPs via hydrogen bonds with aspartic acid-modified SiNPs, as shown in Scheme 1. First, the SiNPs were modified with aspartic acid through conjugation of the hydroxyl group of SiNPs to the amino group of aspartic acid by using PMPI heterobifunctional cross-linking. The SiNPs (20 mg) washed with ethanol were suspended in 2 mL of dimethyl sulfoxide (DMSO). A solution containing 2.1846 mg of PMPI in 150 µL of DMSO was then added to the SiNP suspension and incubated with gentle shaking for 1 h at 37 °C. The PMPI activated SiNPs were washed and resuspended in 2 mL NaHCO₃ solution (pH 8.3). 2 mL of 5 mg/mL aspartic acid was added to 1 mL of 10 mg/mL PMPI activated SiNPs and reacted for 12 h at 4 °C. Then, the mixture of aspartic acid modified SiNPs and aspartic acid was then thoroughly washed to remove uncoordinated aspartic acid. After the SiNPs were modified with aspartic acid, 875 µL of 4 mg/mL insulin was added to the solutions of aspartic acid-capped SiNPs and incubated for 16 h at 4 °C. Following repetitive washing of SiNP–insulin conjugates and removal of unconjugated insulin by centrifugation, the SiNP–insulin conjugates were redispersed in PBS for future usage. The unconjugated insulin presented in the supernatant was determined using the Bradford method. The amount of conjugated insulin was calculated as Formula 2 (31), where \( q \) was the amount of insulin conjugated onto a unit mass of SiNPs (mg/mg). \( W_0, W_a, \) and \( W_{SiNP} \) represented the total amount of insulin added, the amount of insulin in supernatant after conjugation, and the mass of SiNPs, respectively. To determine

Figure 2. (A) RMSC viability in the presence of pure SiNPs of different concentrations from 0 to 2 mg/mL after the treatment at 24, 48, and 72 h, respectively. (B) Cell morphology of RMSCs treated with pure SiNPs at last concentration of (a) 0, (b) 0.05, (c) 0.1, (d) 0.2, (e) 0.3, (f) 0.4, (g) 0.5, (h) 1, and (i) 2 mg/mL, respectively, for 72 h.
if the insulin was conjugated to the SiNPs, the insulin reacted SiNPs were characterized by Fourier transform infrared (FTIR) spectroscopy analysis.

Assessment of RMSC Adipogenic Differentiation with SiNP–Insulin Conjugates. RMSCs were seeded onto six-well tissue culture plates in LG-DMEM growth medium at the density of $2 \times 10^4$ cells/cm$^2$ and cultured to 100% confluence. To assess the adipogenic differentiation of RMSCs treated with SiNP–insulin conjugates, the cells were exposed to four rounds of adipogenic induction with different induction media for three days, with a resting period of one day in different maintenance media, respectively, as shown in Table 1. After treatment by adipogenic induction, the qualitative and quantitative analysis for RMSC adipogenesis was studied as mentioned above. All experiments were carried out more than three times.

RESULTS AND DISCUSSION

Characterization of SiNPs. The SiNPs, FSiNPs and insulin-conjugated SiNPs were first characterized by transmission electron microscopy (JEM-1230). The morphological analysis showed that all of them were well-dispersed spherical nanoparticles and uniform in size (Figure 1a,b,c). The diameters of SiNPs, FSiNPs, and insulin-conjugated SiNPs were 58.7 ± 1.1 nm, 52.6 ± 1.2 nm, and 60.4 ± 1.4 nm, respectively. The insulin-conjugated SiNPs were obtained through the covalent modification of insulin on the surface of SiNPs. The TEM imaging results indicated that the sizes of insulin-conjugated SiNPs were a little larger than that before modification, possibly due to the conjugation of insulin. Additionally, the size distribution of SiNPs before and after bioconjugation has also been determined by dynamic light scattering (DLS) assay using a Malvern Zetasizer (Zetasizer NanoZS). The results showed that the hydrodynamic diameter of SiNPs before bioconjugation was about 141 nm. When the SiNPs were conjugated with insulin, the size of the insulin-conjugated SiNPs was little changed, with diameter of about 153 nm (Supporting Information Figure S1), which was consistent with the size change trends characterized by TEM imaging. While the mean diameters of SiNPs before and after bioconjugation determined by DLS were both larger than those determined by TEM, the reason for this is that the DLS values of nanoparticles in aqueous solution are always larger than solid-state diameters in a monolayer by TEM, as reported in the literature (32).

Effect of Pure SiNPs with Different Concentration on Cell Viability and Proliferation. Our previous biocompatibility studies of SiNPs with cancer cells and normal cells have shown that the bioeffects of SiNPs on cancer cells and normal cells were both concentration-dependent. The cytotoxicity increased with increasing SiNP concentration (33). Therefore, the effect of SiNP concentration on the metabolic activity of RMSCs, a parameter commonly used to reflect cytotoxicity, was first assessed before using SiNPs as carriers of insulin for RMSC adipogenic differentiation. As shown in Figure 2A, addition of SiNPs to the cell culture medium with last concentration of 0.05 mg/mL did not affect the metabolic activity of RMSC cells after exposure for 24, 48, or 72 h. Although the cell survival rate decreased as the concentration of SiNPs increased to 0.1 mg/mL, the survival rate of the cells after exposure for 24, 48, or 72 h remained 90 ± 6%, 84 ± 9%, and 82 ± 5%, respectively. Moreover, the difference in effects observed for the cells treated with either 0.05 mg/mL or 0.1 mg/mL SiNPs was not more than 10% in the three time points. The results indicated that addition of SiNPs to the cell culture medium with last concentrations of 0.05 mg/mL and 0.1 mg/mL did not affect the metabolic activity and proliferation of RMSCs. As the concentration of SiNPs increased, the cell survival rate de-
creased. If the last concentration of SiNPs was not higher than 0.1 mg/mL, the survival rate of the cells after exposure for 24 h remained around 80%. However, the treatment with SiNPs for either 48 or 72 h significantly decreased the metabolic activity of RMSCs at concentrations starting from 0.1 mg/mL. When SiNP concentration was increased to 2 mg/mL, the treatment of RMSCs at 24, 48, or 72 h caused a reduction in metabolic activity to 52 ± 9%, 24 ± 3%, and 23 ± 7%, respectively. These data indicated that the effect of SiNPs on RMSC viability and proliferation was concentration-dependent. With the increase of SiNP concentration, cytotoxicity increased gradually. The SiNPs were biocompatible with RMSCs if the treatment concentration of SiNPs was not more than 0.1 mg/mL.

The morphological changes and cell confluence of treated RMSCs with different concentrations of SiNPs for 72 h were also observed under an inverted phase-contrast microscope. After 72 h exposure to SiNPs with last concentration of either 0.05 or 0.1 mg/mL, the microscopic evaluation of RMSCs showed no major changes in cell morphology and cell confluence as compared to unexposed RMSCs (Figure 2B, a–c). Most cells showed a fibroblast-like morphology, growing in a spindle shape and polygon shape. They displayed eddy current array after 90–100% confluence. However, proliferation of RMSCs has been shown to be inhibited when the RMSCs were treated with high concentrations of nanoparticles starting from 0.1 mg/mL. When SiNP concentration was increased to 2 mg/mL, there was only about 70% confluence (Figure 2B, d). With further increase of SiNPs concentration, the confluence of cells that were involved in fusion was less than 50%, and the cells appeared to retract and showed a greater tendency to die. The
cell toxicity increased obviously. Cell morphology had a clear retraction and the trace of the dead cells separated from the dishes could be easily observed, as shown by the arrow marks in Figure 2B, e–i. The morphological analysis results also confirmed that the effect of SiNPs on RMSC viability and proliferation was concentration-dependent.

After screening the SiNP concentration creating little cytotoxicity on the RMSCs, further experiments were confirmed to investigate if the pure SiNPs with concentration of 0.1 mg/mL had any effect on the cellular ultrastructure, adipogenic differentiation, and osteogenic differentiation.

**Figure 5.** Effect of 0.1 mg/mL SiNPs on adipogenic differentiation of RMSCs. (A) Imaging of oil red-O stained lipid vacuoles of RMSCs. (B) Absorbance intensity of oil red-O stained RMSCs. (a), (b), (c), and (d) represented adipogenic differentiation medium induced SiNP pretreated RMSCs, adipogenic differentiation medium induced RMSCs, RMSCs without adipogenic differentiation medium, and SiNP pretreated RMSCs without adipogenic differentiation medium, respectively.

**Cellular Uptake of SiNPs and Effect of Uptaken SiNPs on Cellular Ultrastructures.** Using confocal laser scanning microscopy, the cellular uptake of SiNPs by RMSCs was determined by the incorporated Rubpy fluorescence dye. Merged images of the red fluorescence from FSiNPs and the blue fluorescence from DAPI stained nuclei indicated that FSiNPs could be effectively taken up by the RMSCs and mainly occupied the cytoplasm after the RMSCs were treated with 0.1 mg/mL FSiNPs for 24, 48, and 72 h, respectively (Figure 3). The RMSCs still maintained good shape and growth conditions after uptake of a large number of particles.
In order to further confirm the uptake of SiNPs by RMSCs and investigate the effect of uptaken SiNPs on cellular ultrastructures, TEM analyses of RMSCs treated with 0.1 mg/mL of SiNPs for 72 h were performed (Figure 4a,b). From the whole-cell images taken, the structures of RMSC cells treated with SiNPs were preserved by comparison with the control group without treatment with SiNPs. The uptaken SiNPs were obviously distributed in the cells and still retained the well-dispersed spherical and uniformity in size after their internalization into cells (Figure 4c,d). The cristae and matrix of the mitochondria were clearly seen (Figure 4d), and the connotations similar to glycogen and cell organs were distributed richly in the SiNP-treated RMSCs (Figure 4e). The nucleolus remained intact with nuclear bags, excrescences, and nucleolus (Figure 4f). As we know, the RMSCs are multipotent stem cells that can differentiate into a variety of cell types in vivo and in vitro. The rich cell organs are the necessary structural and functional units of RMSCs, and the connotations similar to glycogen can provide the energy for differentiation. These cell ultrastructure image results indicated that the treated RMSCs stayed in a relatively active period with normal cell function.

Effect of Pure SiNPs on the Adipogenesis and Osteogenesis of RMSCs. The effect of SiNPs on the ability of RMSCs to differentiate into adipocytes and osteocytes was subsequently examined. For differentiation into adipocytes, RMSCs were seeded onto six-well tissue culture plates in growth medium at $2 \times 10^4$ cells/cm² and exposed to 0.1 mg/mL SiNPs for 24 h. Then, RMSCs were subjected to adipogenic differentiation...
medium for 18 days. Meanwhile, three control groups were designed, including the RMSC adipogenic induction without pretreatment of SiNPs, the RMSCs cultured in the absence of adipogenic stimulants, and the SiNP pretreated RMSCs cultured in the absence of adipogenic stimulants. The intracellular lipid accumulation was determined by examination with light microscopy and absorbance measurement at 490 nm after Oil red-O staining. As shown in Figure 5A, the total amount of accumulated lipids in the SiNP pretreated RMSCs was close to the untreated RMSC group following the 18 days of adipogenic stimulation in the presence of adipogenic stimulants. The absorbance values lacked any significant difference between SiNP-treated RMSCs and untreated RMSCs during adipogenic differentiation (Figure 5B). However, both the RMSCs and SiNP pretreated RMSCs had no lipid vacuoles if they were not induced by adipogenic stimulants. These results revealed that the presence of pure SiNPs did not interfere with adipogenic differentiation of RMSCs.

For differentiation into osteocytes, RMSCs were seeded onto six-well tissue culture plates in growth medium at $3 \times 10^4$ cells/cm$^2$ and exposed to $0.1 \text{mg/mL SiNPs for 24 h}$. Then, RMSCs were subjected to osteogenic differentiation medium for 30 days. Three control groups were also designed, which included the RMSC osteogenic induction without pretreatment with SiNPs, the RMSCs cultured in the absence of osteogenic differentiation medium, and the SiNP pretreated RMSCs cultured in the absence of osteogenic differentiation medium. Alizarin red staining showed that the RMSCs had good biological characteristics of osteocytes in the presence of SiNP treated RMSCs similar to untreated cells following the 30 days of osteogenic stimulation (Figure 6), and both the RMSCs and SiNP pretreated RMSCs were not stained because they were not subjected to osteogenic differentiation medium. The amount of calcium mineralization by the calcium assay results also lacked significant difference between SiNP treated RMSCs and untreated RMSCs following 30 days of osteogenic stimulation, suggesting that the presence of SiNPs had no effect on osteogenic differentiation of RMSCs.

Characterization of SiNP–Insulin Conjugates Using Fourier Transform Infrared (FTIR) Spectroscopy. FTIR spectroscopy was used to determine if the insulin was conjugated to the SiNPs. FTIR spectra of pure SiNPs and pure insulin as
c
control group were also recorded. As shown in Figure 7, a comparison of spectra with that recorded from the pure SiNP samples (curve a) revealed the presence of prominent resonance at $1630 \text{ cm}^{-1}$, which was ascribed to the physically adsorbed water, probably in the micropores ($34$). The determination of spectra with that recorded from insulin-conjugated SiNPs samples (curve b) showed the presence of prominent resonance at $1540 \text{ cm}^{-1}$. This absorption band absent in FTIR spectra recorded from the SiNPs samples was identified as the amide II vibrational mode, suggesting that insulin was indeed conjugated to the SiNPs. Insulin is a very important biologically active protein composed of 51 amino acids. Each insulin protein had strong absorption bands at $1540 \text{ cm}^{-1}$ and $1640 \text{ cm}^{-1}$ (curve c), which were identified as the amide I and II vibrational modes, respectively. The position of these two bands was important to the biological activity of insulin protein. The absorption band at $1640 \text{ cm}^{-1}$ (amide I) recorded from the insulin-conjugated SiNP samples should be overlap the spectra of SiNPs and insulin. The amount of conjugated insulin on the SiNPs was $0.3437 \text{ mg/mg calculated from Formula 2}$.

Effect of Insulin-Conjugated SiNPs on the Adipogenic Differentiation of RMSCs. To induce the RMSC adipogenic differentiation in vitro using defined culture milieu, the adipogenic differentiation medium should be complete. Insulin is one of the important supplements of adipogenic induction and adipogenic maintenance media. In the present study, the insulin-conjugated SiNPs for RMSC adipogenic differentiation have been investigated. After culturing in a different culture medium for a period of 18 days, oil red-O staining of the lipid vacuoles of RMSCs was carried out. As shown in Figure 8A, the RMSCs had plenty of lipid vacuoles after induction with complete adipogenic differentiation medium, which indicated the adipogenic differentiation of RMSCs in vitro. When the insulin in the complete adipogenic differentiation medium and maintenance media were both replaced with equal-quantity insulin conjugated SiNPs, the RMSCs also had plenty of lipid vacuoles. However, very few lipid vacuoles were observed for the RMSCs exposed to the adipogenic medium absent of insulin or the adipogenic medium without insulin but with $0.1 \text{ mg/mL pure SiNPs}$. Figure 8B showed the quantitative investigation of oil red-O staining for lipid vacuoles of RMSCs cultured in a different culture medium for the period of 18 days. There were no significant differences in the absorbance values for the RMSCs exposed to the complete adipogenic differentiation medium and the insulin conjugated SiNP adipogenic differentiation medium after $18 \text{ d of culturing}$. Low absorbance values were observed for the RMSCs exposed to the adipogenic medium without insulin but with $0.1 \text{ mg/mL pure SiNPs}$. These results obviously demonstrated that insulin is necessary for the adipogenic differentiation of RMSCs in vitro. The adipogenic differentiation medium without insulin could not induce the adipogenic differentiation of RMSCs even though pure SiNPs had been added. When the insulin was conjugated to SiNPs, the biological activity of insulin as a supplement of the adipogenic differentiation medium was still retained.

As we all know, the successful release of biomolecules from the nanoparticles is critical for nanoparticle-based carriers to meet specific needs. The release of the conjugated biomolecules from the nanoparticles is related to many factors, including the materials of the nanoparticles, the interaction of nanoparticles with biomolecules, and so on. In this work, the nature of interaction between insulin and SiNPs carriers would be expected to play a major role in the release of insulin. As described above, insulin was linked to the aspartic acid—SiNP surface coating via much weaker hydrogen bonding. In this case, the release of insulin is more facile and faster in the biological system as reported previously ($35$). Therefore, the release of
insulin from SiNP conjugates would be possible after uptake by the RMSCs. Then, the potential release characteristic made the SiNPs able to be used successfully as biocompatible carriers of insulin for RMSC adipogenic differentiation.

The aforementioned cell viability assay of SiNPs with RMSCs and the in vitro cell studies of insulin-conjugated SiNPs on the adipogenic differentiation of RMSCs have clearly demonstrated that SiNPs could be used as biocompatible carriers of insulin for RMSCs adipogenic differentiation. The SiNP-based carriers are expected to keep the biomolecules’ activity and stability. It is conceivable that this novel nanocarrier for the intracellular and controlled delivery of bioactive molecules can likely be an excellent candidate for modulation of the cellular functions in a more effective manner ex vivo and maintenance of the cellular phenotype in vivo upon reimplantation. If substituting the insulin to suit other biomolecules, it is believed that the SiNP based carrier holds great potential application in other stem cell directed differentiation in vitro and in vivo.

**CONCLUSION**

In this work, we demonstrated the biocompatible silica nanoparticle—insulin conjugates for RMSC adipogenic differentiation. The biocompatibility of the SiNPs with RMSCs was first investigated, namely, for cell viability, proliferation, cellular uptake, cellular ultrastructures, adipogenic differentiation, and osteogenic differentiation. Results showed that the SiNPs with concentration creating little cytotoxicity on the RMSCs could be well uptaken by RMSCs and would not affect the cellular ultrastructures, RMSC adipogenic differentiation, and osteogenic differentiation. Further experiments demonstrated...
that insulin could be conjugated to the SiNPs and retain its biological activity for induction of the adipogetic differentiation of RMSCs in combination with other supplements. Therefore, SiNPs could be used as biocompatible intracellular nanocarriers of biological agents able to modulate the behavior of stem cells.

ACKNOWLEDGMENT

This work was partially supported by Program for Innovative Research Team of Hunan National Science Foundation (10JJ7002), Program for New Century Excellent Talents in University (NCET-06-0697) and National Science Foundation of P.R.China (90606003, 20775021).

Supporting Information Available: Characterization of SiNPs and SiNP–insulin conjugates by dynamic light scattering (DLS). This material is available free of charge via the Internet at http://pubs.acs.org.

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