

**TOXICITY OF SILVER NANOPARTICLES IN MOUSE EMBRYONIC STEM
CELLS AND CHEMICAL BASED REPROGRAMMING OF SOMATIC CELLS
TO SPHERE CELLS**

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ABSTRACT

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ABSTRACT 1: Silver nanoparticles (Ag Np's) have an interesting surface chemistry and unique plasmonic properties. They are used in a wide variety of applications ranging from consumer products like socks, medical dressing, computer chips and it is also shown to have antimicrobial, anti bacterial activity and wound healing. Ag Np toxicity studies have been limited to date which needs to be critically addressed due to its wide applications. Mouse embryonic stem (MES) cells represent a unique cell population with the ability to undergo both self renewal and differentiation. They exhibit very stringent and tightly regulated mechanisms to circumvent DNA damage and stress response. We used 10 nm coated (polysaccharide) and uncoated Ag Np's to test its toxic effects on MES cells. MES cells and embryoid bodies (EB's) were

treated with two concentrations of Ag Np's: 5 µg/ml and 50 ug/ml and exposed for 24, 48 and 72 hours. Increased cell death, ROS production and loss of mitochondrial membrane potential and alkaline phosphatase (AP) occur in a time and a concentration dependant manner. Due to increased cell death, there is a progressive increase in Annexin V (apoptosis) and Propidium Iodide (PI) staining (necrosis). Oct4 and Nanog undergo ubiquitination and dephosphorylation post-translational modifications in MES cells thereby altering gene expression of pluripotency factors and differentiation of EB's into all the three embryonic germ layers with specific growth factors were also inhibited after Ag Np exposure. Flow cytometry analysis revealed Ag Np's treated cells had altered cell cycle phases correlating with altered self renewal capacity. Our results suggest that Ag Np's effect MES cell self renewal, pluripotency and differentiation and serves as a perfect model system for studying toxicity induced by engineered Ag Np's.

ABSTRACT 2: The reprogramming of fibroblasts to pluripotent stem cells and the direct conversion of fibroblasts to functional neurons has been successfully manipulated by ectopic expression of defined factors. We demonstrate that mouse fibroblasts can be converted into sphere cells by detaching fibroblast cells by proteases and then using AlbuMAX I-containing culture medium without genetic alteration. AlbuMAX I is a lipid-rich albumin. Albumin-associated lipids arachidonic acid (AA) and pluronic F-68 were responsible for this effect. The converted colonies were positive for both alkaline phosphatase and stage specific embryonic antigen-1 (SSEA-1) staining. Global gene expression analysis indicated that the sphere cells were in an intermediate state compared with MES cells and MEF cells. The sphere cells were able to differentiate into tissues

representing all three embryonic germ layers following retinoic acid treatment, and also differentiated into smooth muscle cells following treatment with vascular endothelial growth factor (VEGF). The study presented a potential novel approach to transdifferentiate mouse fibroblast cells into other cell lineages mediated by AlbuMAX I-containing culture medium.

Dedicated to Krishna

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LIST OF ABBREVIATIONS AND NOTATIONS

ABBREVIATION	DEFINITION
Ag Np's	Silver Nanoparticles
MES	Mouse embryonic stem cells
MEF	Mouse embryonic fibroblasts
KSR	Knockout Serum medium replacement
bFGF	Basic fibroblast growth factor, FGF-2
BMP-4	Bone morphogenetic protein-4
TGF- β 1	Transforming growth factor β 1
HGF	Hepatocyte growth factor
β -NGF	β nerve growth factor
RA	All Trans Retinoic acid
EGF	Epidermal growth factor
α -SMA	Alpha-smooth muscle actin

CHAPTER I

LITERATURE REVIEW

INTRODUCTION

Two separate areas of research are focused in this manuscript, the first showing the effects of Ag Np's on mouse embryonic stem (MES) cell fate and its pluripotency factors and the second showing the conversion of MEF cells to sphere cells induced by Albumax I containing medium which share similar characteristics to MES cells including pluripotency factor expression and differentiation potential to all the three embryonic germ layers. In this chapter, I will present a literature review of nanotechnology, nanoparticles and types, its characteristics, synthesis, applications followed by reviewing Ag Np applications in the biomedical field and its toxic effects on biological models. I will end by presenting the objectives and significance of my research.

Nanotechnology

Nanotechnology can be defined as a branch of engineering, science and technology dealing with nanoscale and ultrafine objects in a range between 1 - 100 nm in dimension. The shape and size of these nanomaterials can be controlled thereby finding its way in a wide range of applications (Beer, Foldbjerg, Hayashi, Sutherland, & Autrup, 2011).

The devices, systems and materials exhibit biological, physical and chemical properties (Assadi, Afrasiabi, Nabipour, & Seyedabadi, 2011). Nanotechnology is projected to be a \$1 trillion industry by 2015 according to the National Nanotechnology Industry (NNI). The expected materials made by nanomaterials are capable of being stronger, more durable, lighter, cheaper and cleaner which are due to its characteristics that include high activity, providing a catalytic surface, high surface area, prone to agglomerate, adsorbent, and showing a range of chemistries (Chekman et al., 2011; Gopinath, Gogoi, Chattopadhyay, & Ghosh, 2008). Wide varieties of nanomaterials are in use and can be classified into one, two and three dimensional nanomaterials. Nanomaterials can also be classified into three major types, namely (1) naturally occurring nanoparticles that can be found in space dust, volcanoes, and forest fires (2) anthropogenic or incidental nanoparticles that occur as byproducts from industries or from exhaust due to combustion and (3) engineered nanoparticles made intentionally for its potential applications (Lynch, Salvati, & Dawson, 2009). One dimension nanomaterials include thin films. Two dimensional nanomaterials are carbon nanotubes, nanowires, biopolymers and inorganic nanotubes and three dimensional nanomaterials include nanoparticles, fullerenes, quantum dots and dendrimers. The general process of manufacturing nanoparticles include laser ablation, plasma synthesis, combustion, arc method, electrolysis, pyrolysis, diffusion flame synthesis, chemical precipitation and vapor deposition and mechanical processing, wet phase processing and high energy ball milling (M. H. Kim et al., 2011).

Types of Nanomaterials

Carbon based nanomaterials

Carbon Black: Carbon black is used in inks, toners, paper manufacturing industry, coatings, fillers for tyres, fibers, pipes and plastics that can have conducting or insulating properties. It has also made its way into marine and aerospace technologies due to its protection from UV light, pigmentation, and conductivity (Ansari & Husain, 2011). Its frequent use in daily applications makes it one of the largest productions in engineered nanomaterials (Bendrea, Cianga, & Cianga, 2011).

Graphite: Graphite is a one dimensional carbon having novel magnetic properties. It is used as a lubricant and reduces friction between two surfaces coated with graphite material because of its nano-sized spacing and sub-nano thickness (Bendrea et al., 2011; Dorrer & Ruhe, 2011). It can also be used as a storage space for hydrogen and can be used in hydrogen fuel cells (Paull, Wolfe, Hebert, & Sinkula, 2003).

Carbon nanotubes: They are two dimensional nanomaterials having interesting electrical and mechanical properties by exhibiting efficient conducting capacity, tensile strength, elastic modulus and low density which makes it one of the strongest materials known to man (Valentini, Amine, Orlanducci, Terranova, & Palleschi, 2003; Yeom, Kang, Kim, & Kang, 2011). Single and multi walled forms are the most common types of carbon nanotubes. Single walled forms share better electrical properties and can therefore be used in electrical wiring systems where as multi walled forms can slide into one another making it stronger. Multi walled carbon nanotubes are be used in sensors, display devices, semiconductor devices, energy storage devices and other nanoelectronic products. Since functional groups can be added to carbon nanotubes, they can also be

used in many biomedical devices like prosthetics and surgical implants, adhesives, connectors, fabrics, ceramics, thermal materials and air, water and gas filtration systems (Wiechers & Musee, 2010; Youns, Hoheisel, & Efferth, 2011).

Carbon buckyballs: Carbon buckyballs or buckminsterfullerene are fullerenes having 60 carbon atoms arranged in the shape of a geodesic dome. It finds its applications in electronic circuits, drug delivery systems, chemical sensors, optical devices, surface lubricants and optical devices (Ansari & Husain, 2011; Majestic et al., 2010).

Inorganic nanotubes: They are two dimensional materials which include titanium dioxide, tungsten and molybdenum sulphide, and boron nitride. Their unique properties include resistance to shockwave impacts, lubrication, hydrogen and lithium storage and catalytic activity (Veerapandian & Yun, 2011). They are used in energy storage devices like rechargeable batteries, photo catalysis, sporting goods, and chemical sensors (Liu, Qiao, Hu, & Lu, 2011).

Metals: Metal nanoparticles include cobalt, silver, iron, aluminum, nickel, gold and copper. Aluminum being a highly reactive metal is used in explosives and is stable in air. Iron on the other hand has a large surface area and high reactivity and is used in detoxification processes, pesticide control, chlorinated solvents, and soil remediation. Cobalt has magnetic behavior and therefore can be used in many medical imaging diagnostics (Ansari & Husain, 2011).

Metal Oxides: Metal oxides comprise the largest group of inorganic nanomaterials. They include oxides of titanium, silicon, zinc, aluminum, iron and cerium. TiO_2 and ZnO_2 (to a lesser extent) are mainly used in paints due to its protection from UV light and paper making. They are used in cosmetics along with Al_2O_3 , sunscreens and plastics, and

solarcell technology. Addition of silica to materials helps as a filling agent due to its viscous properties and is used in sealants, cements, pharmaceuticals and abrasives (Wiechers & Musee, 2010; Youns et al., 2011). Iron and cerium oxide are used as catalyst for increasing combustion and oxidation of diesel fuels.

Quantum Dots: Quantum dots are portions of semiconductor nanocrystals. They have narrow emission spectra with brighter emission and are used in labeling and detection in the field of biomedicine and imaging (Ahamed, Alsalhi, & Siddiqui, 2010). Light emitting diodes, multi colored lasers, solar cells, transistors and organic dyes use quantum dots due to its sensitivity and specificity.

Dendrimers: Dendrimers are three dimensional, large and complex polymeric molecules having a near perfect polymeric shape. They can be used as carrier molecules that are biologically active, drug delivery, therapeutic agents, gene therapy, and catalysis (Zhang, Yu, Colvin, & Monteiro-Riviere, 2008).

Toxic Effects of Nanoparticles on Biological Systems

Nanoparticles utilization in industries and consumer products are ever increasing. This has raised alarming concerns about the toxic effects nanoparticles have on human health. In the recent past, many studies have shown nanoparticles to actively interact with pro and eukaryotic cells (mammalian cells). ZnO, TiO₂, CuO and Co nanoparticles have shown to be toxic in human HepG2 (liver carcinoma) cells (Y. Wang et al., 2011). They show increased ROS generation with altered mitochondrial membrane permeability and eventually cell death not only induced by ROS alone, but due to active degradation of cell and organelle membranes. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium

bromide, a yellow tetrazole) assay showed that these cells had reduced metabolic activity. Of these, CuO Np's showed to be the most toxic followed by ZnO, Co and TiO₂ (Creton, Zhu, & Gooderham, 2005).

Toxic Effects of Silver Nanoparticles on Biological Systems

Ag Np's are used in many commercialized products compared to any other nanoparticles made today (Ahamed et al., 2010). They are used in paints, as food preservatives, surgical and medical instruments and imaging, socks, medical dressing, computer and other electronic chips and devices, fabrics, disinfectants, wound dressings, bandages, nanofiber mats, creams and ointments, catheters, and prostheses (Ahamed et al., 2010; Ansari & Husain, 2011). It is also used in the treatment of water purification. Therefore exposure of Ag Np's to human health can cause potential and serious health risks which could be acute or chronic depending on the types and sources (Asharani, Lianwu, Gong, & Valiyaveetil, 2011). At the same time, Ag Np's have shown to have bactericidal activity, and increase the bioavailability of the drug to the body (Asharani, Hande, & Valiyaveetil, 2009). The small size, high surface area, high rates of effective collision makes it highly reactive to biomolecules (Beer et al., 2011). Since the surface of Ag Np's can be easily functionalized or coated with polysaccharides thereby changing its reactive properties, they are used for drug stabilization and targeted delivery to treat many diseases including malaria, cancer, tetanus, typhoid, tuberculosis and lupus (Ansari & Husain, 2011). They are also shown to inhibit the binding of HIV-1 virus to host cells in vitro (Elechiguerra et al., 2005). Due to continuous exposure of Ag Np's, they have shown to accumulate in organs like liver, lungs and brain in Rats and Mice, cause genetic

abnormalities and malfunctions during development in *Drosophila*, mice and Zebrafish (Comfort, Maurer, Braydich-Stolle, & Hussain, 2011). Since Np's cannot be metabolized, they tend to stay in the body for longer time periods. The degree of ionization, stereochemistry, solubility, oxidation-reduction potentials, intermolecular forces and intermolecular distances between functional groups affect/alter the interaction of Ag Np's with cells and biomolecules (Ma, Lu, & Huang, 2011). It has also been shown that small Ag Np's are more toxic than large Ag Np's (Ahamed et al., 2010; Ansari & Husain, 2011). Exposure to Ag Np's present in the environment accumulates in the liver and brain which increases stress responses in alveolar macrophages, lung epithelial cells and a decrease in dopamine secretion in neuro endocrine cells and additional accumulation in the lungs and kidneys (Kang, Ryoo, Lee, & Kwak, 2011). Under non toxic concentrations (<0.5 µg/ml), Ag Np's can be toxic in human hepatoma cells by increasing intracellular ROS production followed by an increase in p53. But toxic concentrations (>1 µg/ml) showed abnormal cellular morphology, cell shrinkage and increased micronucleus formation and chromosomal damage (Kawata, Osawa, & Okabe, 2009). Many studies have found topical dressing containing silver being toxic to keratinocytes and fibroblasts (Deng et al., 2010; Lee et al., 2011). It has the potential to react with enzymes involved in metabolism, thereby having drastic effects on energy production. Human exposure to silver and compounds of silver mainly take place through three different routes: Dermal, oral and inhalation (Dorrer & Ruhe, 2011). Chronic exposure of silver to the body can have considerable accumulation in the liver and lungs. Due to prolonged usage of contraceptive devices and related products, silver can be toxic to germ line stem cells (Braydich-Stolle et al., 2010). Alterations in ROS and glutathione activity, MMP, cell

viability and apoptosis are common processes of Ag NP induced stress mechanisms. Also, Ag NP's cause an increase in protein expression p53, a tumor suppressor, p21, Noxa, Bax and DNA damage repair proteins RAD 51 and H2AX (Zhu, Chang, Dai, & Hong, 2007). Epidermal growth factor signaling was disrupted along with increased ROS production, decreased Akt and Erk phosphorylation, cell migration and receptor expression when human epithelial cells were exposed to 10 nm Ag NP's. Spermatogonial stem cells showed decreased cell proliferation due to disruption in glial cell line derived neurotrophic factor and Fyn kinase (Wiechers & Musee, 2010). Nrf2 (NF-E2-related factor 2), which is a transcription factor that binds to antioxidant response elements and 8-Oxoguanine DNA glycosylase 1 (OGG1) showed decreased protein expression and heme oxygenase - 1, a cytoprotectant along with PI3K and p38 MAPK signaling activity was increased in ovarian carcinoma cells exposed to nano silver (Wiechers & Musee, 2010; Yeom et al., 2011; Zhang et al., 2008). Cell membrane integrity, and metabolic activity was altered in murine fibroblasts (L929) and increased ROS, pro inflammatory markers including tumor necrosis factor - α (TNF - α), granulocyte colony stimulating factor (G-CSF) and macrophage inflammatory proteins (MIP's) in macrophage cell line (RAW 264.7) was observed due to Ag Np toxicity (Y. Wang et al., 2011). In Mice, cytokines IL-1, IL-4, IL-6, IL-10, IL-12 and transforming growth factor - β (TGF - β) were increased in a dose dependent manner in addition to increased B cell distribution and Ig E production and Rat lung epithelial cells (R3/1) showed decreased lactate dehydrogenase activity after Ag Np exposure. Nano silver causes histopathologic abnormalities in spleen, liver and skin including hepatic cord deformation, hepatocyte degeneration, overproduction of Kupffer cells, thinning of the red capsule, red pulp

inflammation and white pulp atrophy in the spleen, decreased collagen fiber production, lamina propria attachment and thickness of papillary zone layer and epidermis, increased Langerhans cells production along with inflammation and collagen levels in the skin of Pigs (Singh & Nalwa, 2011; Veerapandian & Yun, 2011; Y. Wang et al., 2011). Global gene expression profiling in human dermal fibroblasts indicated changes in gene expression patterns concerned with cell cycle arrest, DNA damage, energy metabolism and cytoskeleton disruption. Gap junctions are known to allow passage of small molecules in between neighboring cells thereby regulating tissue maintenance and organ homeostasis. Ag NP's increase intercellular transport of molecules between cells by over expressing Cx43, increasing LDH release, induces cell cycle arrest in G2/M and sub G1 phases leading to cell death by protein kinase C- ζ (PKC- ζ) down regulation in human lung adenocarcinoma cell line A549 (Deng et al., 2010). In human lung fibroblasts (IMR-90) and human glioblastoma cells (U251), the main route of entry of Ag NP's into cells were through clathrin dependent endocytosis and macropinocytosis. Mitotic arrest and chromosomal instability were seen in these cells. Further Ag NP's decreased proliferation of U251 cancer cell line, and down regulated filamin which cross link microfilaments and stabilizes the cytoskeletal framework followed by altered intracellular Ca^{+2} transients. After uptake of Ag NP's, they are usually found in the distributed in the peri nuclear region and in endo-lysosomes in human mesenchymal stem cells. There was decreased chemotactic activity, IL-6 and vascular endothelial growth factor (VEGF) expression at non toxic concentrations (<1 $\mu\text{g/ml}$), but was increased when exposed to cytotoxic concentrations (10 $\mu\text{g/ml}$) (Asharani et al., 2009; Foldbjerg et al., 2009; Greulich, Kittler, Epple, Muhr, & Koller, 2009). IL-8 on the other hand was significantly increased which

induced cell activation of human mesenchymal stem cells. Human peripheral monocytes also showed increased cell proliferation, and CD 54 over expression after Ag Np uptake. Further the particles were distributed throughout the cytoplasm and there was a dramatic increase in ROS by 6 hrs in THP-1 human monocytes (Foldbjerg et al., 2009). In contrast, T- lymphocytes did not exhibit increased cell proliferation. These studies do suggest that Ag Np distribution in cells depends on the type of cells and the mechanism of uptake into the cells. Rat endothelial cells which are required for angiogenesis showed contrasting results where low concentration of Ag Np inhibited cell proliferation and vasoconstriction to impair NO signaling and exposure to high concentrations increased proliferation and vasorelaxation (Ma et al., 2011; Mailander & Landfester, 2009). Bovine retinal endothelial cells showed decreased cell proliferation and migration when treated with Ag Np's. The concentration of Ag Np's used in these studies ranged from <1-200 µg/ml with most of the studies using an average of 5-50 µg/ml and the diameter ranged between 10-100 nm (Kawata et al., 2009; S. W. Kim, Nam, & An, 2011; Paull et al., 2003).

Non mammalian models including Zebra fish (*Danio Rerio*), Fruit fly (*Drosophila melanogaster*), Rainbow trout fish (*Oncorhynchus Mykiss*), *Caenorhabditis elegans*, and Fathead minnows show abnormalities in the developing embryos, an increase in oxidative stress, DNA damage response, metallothionein (which prevent abnormal metal catalyzed reactions), altered development of the brain, liver and lungs and an overall increase in apoptosis (S. W. Kim et al., 2011; Korani, Rezayat, Gilani, Arbabi Bidgoli, & Adeli, 2011; Kumari & Yadav, 2011; Loeschner et al., 2011).

Mouse Embryonic Stem Cells as a Model System

MES cells are derived from the inner cell mass present in the blastocyst of the developing embryo (Boyer et al., 2005; Brambrink et al., 2008). They are clonal cell lines isolated from pre-implantation embryos of mice. The main characteristics include indefinite replication/immortality, self renewal, pluripotency and differentiation into all the 3 germ layers namely the ectoderm, endoderm and mesoderm. The ectoderm mainly comprises of nervous tissue and skin, endoderm gives rise to the lung, esophagus, liver, pancreas, and intestine while the mesoderm mainly gives rise to hematopoietic (Barrand & Collas, 2010; Boyer et al., 2005) stem cells and mesenchymal stem cells which further differentiate to different blood and bone marrow cells. They can be easily culture *in vitro* by well defined culture conditions due to their rapid proliferation which is used to determine the cell's replicative capacity (Chan, Yang, & Ng, 2011). On the other hand, somatic cells do not proliferate indefinitely *in vitro* and are restricted to certain cell divisions giving rise to cell senescence and replication arrest. MES cultures *in vitro* give rise to EB's which have the capacity to differentiate into all the germ layers. Since pluripotent MES cells can give rise to most of the cell types present in the body, the mechanism involved in DNA repair are very stringent and vigorous compared to monopotent somatic cells there by forcing these cells beyond the threshold limit to prevent apoptosis and cell death (Chambers et al., 2007; Chao et al., 2000). Pluripotent MES cells are very important for normal embryonic development. Many DNA damage response signaling pathways are activated depending on the extent of toxic insult to the cells as they are very sensitive to genotoxic agents. MES cells which cannot repair

damaged DNA get eliminated by apoptosis so that the abnormal/mutated MES cells do not interfere or proliferate during normal embryonic development leading to embryo toxicity. Therefore MES cells serves as an excellent model for rapidly assessing toxicity of nanoparticles (Chen, Vega, & Ng, 2008; Chuykin, Lianguzova, Pospelova, & Pospelov, 2008).

Tests to confirm Pluripotency of MES Cells

- Gene expression of Oct4 and Nanog very closely determine the pluripotency of MES cells. Slight fluctuations, especially in Oct4 protein level expression can alter the fate of MES cells to differentiate into embryonic endoderm or trophoctoderm lineages. Nanog, Sox2 and Oct4 cooperate and regulate several target genes involved in embryonic development and lineage commitment.
- Colony formation and rapid proliferation determine the health of MES cells.
- Examining chromosomal number (Karyotyping) and mutation detection. MES cells maintain an exact copy of chromosomes with almost no genetic alterations.
- Determining specific cell surface markers exclusively expresses by MES cells.
- Formation of EB's and capacity to differentiate into all 3 germ layers. MES cells are capable of directed differentiation where in they respond to specific signaling molecules which alter MES cell fate and gene expression.
- Formation of teratomas (benign tumors) when MES cells are injected into nude mice whose immune system is suppressed or genetically modified.
- MES cells have a longer S (Synthetic) phase in the cell cycle compared to somatic cells and can be determined by analyzing cell cycle genes, DNA proliferation and DNA content.

- MES cells exhibit bivalent domains in regions of certain genes by histone modifications, especially through histone 3 lysine 4 methylation (H3K4) for gene activation and histone 3 lysine 27 methylation (H3K27) for gene inactivation. Depending on environmental cues, MES cells either activate or repress these genes rapidly.

DNA Damage Response in MES Cells

Maintaining genomic integrity is an important priority for rapidly dividing MES cells.

They have a very high capacity to resist DNA damage and repair damaged DNA, but at the same time, they are very sensitive to stress and prefers to trigger apoptotic elimination in cells which are irreparable and pose as a threat for future embryonic development (Chuykin et al., 2008; Creton et al., 2005). To protect genome integrity in MES cells, 3 possible mechanisms have been hypothesized:

- Suppression of recombination and mutation between homologous chromosomes
- Increased apoptotic rate
- Use of high fidelity homology mediated repair of double strand breaks

As seen in somatic cells, MES cells do not have a G1 check point and therefore the mechanisms governing G1/S blockage during DNA damage is compromised in MES cells. The two major mechanisms are ATM mediated p53 phosphorylation and arrest in G1 phase and ATM mediated cdk2 phosphorylation mediating G1/S arrest. MES cells accumulating DNA damage will now progress into the S phase of the cell cycle (due to lack of G1 phase) thereby increasing DNA content and the extent of DNA damage which eliminates these cells through apoptosis (DeWeese et al., 1998; Filion et al., 2009). This

mechanism eliminates MES cells with damaged DNA and restores normal cell population during embryonic development. Also, mitotic recombination and mutation frequencies in MES cells are a 100 times lower than in somatic cells. Wild type p53 in MES cells are poorly activated and are mostly localized to the cytoplasm of the cell even after DNA damage (Fan et al., 2011). But with prolonged exposure to stress response, p53 is known to enter the nucleus and suppress Nanog promoter and inducing differentiation and elimination of cells harboring damaged DNA. ATM and γ H2AX activation seems to be the initial step for initiating DNA repair response machinery in MES cells (Fujita et al., 2008). Antioxidants SOD and GPX2 are over expressed in MES cells compared to somatic cells which protects the cells from initial damage due to ROS production. MES cells divide at a rapid rate exhibiting a cell cycle of approximately 10-12 hrs and most of the cells are seen in the S phase (60-70%). There is incomplete maturation of replication forks due to increased DNA replication and showed non-induced single strand breaks (Glinsky, 2008). Although γ H2AX was found in these loci, they did not seem to elicit a DNA damage response, but instead forced the cells to enter S phase and induce apoptosis. Increased single and double stranded breaks, ATM, γ H2AX and p53 can contribute towards eliminating MES cells through apoptosis. S phase cells with γ H2AX have shown to activate GABA receptors in the cell surface of cells and GABA secretion will inactivate these cells which further inhibit cell proliferation (Hackenberg et al., 2011; Hong, Cervantes, & Stambrook, 2006).

Regulators of Pluripotency

Transcription factors Oct4, Nanog and Sox2 form a closed interacting network to regulate pluripotency in MES cells. They are known to be master regulators which control self

renewal and differentiation and proliferation. Oct4 and Nanog are exclusively found in MES cells and their expression drastically decreases when pluripotent stem cells start to differentiate towards specific lineages (Furusawa et al., 2006). Also, additional transcription factors are known to play an important role in maintaining the pluripotent stage of MES cells (Table 1).

Oct4: Octamer binding protein 4, a 352 amino acid protein in mice is known to be the central molecule in the gene regulatory network which supports pluripotency. It belongs to class V POU family of transcription factors (Hong & Stambrook, 2004; Jung et al., 2010). Oct4 is a transcription factor expressed mainly in pluripotent stem cells and their levels are tightly regulated by other transcription factors and post-translational modifications during embryonic development. Down regulation of Oct4 protein (approximately a 2-fold change) *in vivo* in ES cells can lead to differentiation into the trophoblast lineage and up regulation will result in spontaneous differentiation into primitive mesoderm and endoderm lineages (Kang et al., 2011; Loh et al., 2006). Oct4 is the earliest transcription factor which is known to be crucial during pre-implantation development in mice. Human and mouse Oct4 is 87% identical and Bovine and mouse are 81.7% identical in sequence similarity. Over expression or misexpression of Oct4 can change the fate of ES cells to differentiate into other cell types. Oct4 has also been shown to be over expressed in many metastatic tumors including testicular tumors, non small cell lung cancer, primary germ cell tumors, bladder, prostate and urothelial cancer (Masui et al., 2007; Matoba et al., 2006). Recent studies have shown Oct4 to be critical for survival and knockdown of Oct4 increases apoptosis in MES cells (Guo, Mantel, Hromas, & Broxmeyer, 2008).

Nanog: Nanog is a homeodomain containing protein expressed in ES cells and embryonic germ (EG) cells. The homeodomain separates the N and C terminal domains and the C domain acts as the transactivation domain. Nanog is highly conserved across mammalian species (Tay, Zhang, Thomson, Lim, & Rigoutsos, 2008; van den Berg et al., 2008). It has been shown that higher levels of Nanog in ES cells are beneficial for pluripotency, but not in case of Oct4 levels. Nanog is regulated by Oct4 and Sox2 as it contains binding sites POU and Sox elements for its regulation and expression. Nanog is also regulated by the levels of LIF and STAT3 for maintaining ES cell pluripotency (Miyanari & Torres-Padilla, 2010). The tumor suppressor protein p53 negatively regulates Nanog and promotes differentiation of ES cells during stress conditions. Oct4, Nanog and Sox2 regulate the state of pluripotency in ES cells (Kashyap et al., 2009). Expression of Nanog fluctuates in MES cells as down regulation of Nanog predisposed the cells to differentiate without commitment. Phosphorylation stabilizes expression of Nanog by suppressing ubiquitination. Therefore dephosphorylation makes Nanog unstable and susceptible for degradation resulting in loss of pluripotency (Chao et al., 2000; Do et al., 2009).

Sox2: Sox2 (SRY related HMG box gene 2) is another transcription factor involved in governing pluripotency by interacting with Oct4 and Nanog. Sox2 belongs to the HMG (high mobility group) family of proteins as it contains the SOX box (SRY box containing gene) (Card et al., 2008; Yuan et al., 2010). The HMG domain helps in DNA bending at large angles to assist interaction with other factors (Yi, Pereira, & Merrill, 2008; You et al., 2009; Zhou, Chipperfield, Melton, & Wong, 2007). Phosphorylation of Sox2 increases its transcriptional activity and directly cooperates with Oct4 and to a lesser

extent with Nanog in ES cells. Forced expression of Oct4 can rescue pluripotency in Sox2 null ES cells (Tomioka et al., 2002; Z. X. Wang et al., 2007). Therefore Sox2 is involved in stabilizing ES cell pluripotency by maintaining necessary levels Oct4 expression (Magnani & Cabot, 2008; Masui et al., 2007; Sonne et al., 2010).

The interaction of Ag Np's on MES cells can alter the gene regulatory network of Oct4, Nanog and Sox2. Since these proteins are abundant in MES cells, the influence of Ag Np's on regulation of these genes can act as an indicator for toxicity.

RESEARCH OBJECTIVES

The objectives of this project was to develop an assessment protocol for determination of the toxicity of engineered Ag Np's and understand the effect of Np's on stem cell fate and pluripotency. Many studies have used various somatic cells including lung cells, macrophages, blood cells, skin cells and neurons to study nanoparticles induced toxicity. Of late, the use of adult multipotent stem cells for toxicity assessment has increased due to its potential to give rise to specific lineage of cells. We have used pluripotent MES cells as a model system to measure and study the impact of Ag Np's since it very sensitive to genotoxic insults and its importance in tissue regeneration.

The main goals of this study are:

- To characterize prolonged exposure (24hrs, 48hrs and 72 hrs) of coated and uncoated Ag Np (5 and 50 µg/ml) induced MES cell toxicity by studying ROS production, cell morphology and colony formation, apoptosis assay, mitochondrial membrane potential, and Alkaline Phosphatase ES cell marker expression.
- To identify changes in gene expression and post-translational modifications induced by Ag Np's in the stem cell pluripotency factors Oct4, Nanog and Sox2
- Whether Ag Np's affect the differentiation potential of MES cells and EB's.
- To analyze MES cell self renewal capacity by cell cycle progression and replication through flow cytometry.

SIGNIFICANCE

Ag Np's are widely used in many consumer products, aerospace materials, electronic chips, and in the medical industry, as an anti microbial agent, bandages and ointments due to its wound healing capacity, surgical masks and instruments, water purification and treatment. Since the nanotechnology industry has progressed significantly in the recent past, the study of toxic effects of nanoparticles on biological systems is limited. A standard model system to test toxicity has not been established. Our study shows that MES cells can be used as an excellent model to assess nanoparticles toxicity in a very quick and efficient way. To our knowledge, this is the first study to use both coated and uncoated Ag Np's and address the differentiation potential, cell death, toxicity and changes in the dynamics of MES cell pluripotency regulators over longer time periods (24 , 48 and 72 hours). The pluripotency regulators of MES cells regulate most of the early developmental genes and lineage commitment genes and we show that Ag Np's have an impact on these regulators and alter the fate of the cell by undergoing post-translational modifications. The results obtained not only open doors to further explore regulation of stem cell renewal in stem cells, but also its involvement in regulating stress response.

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CHAPTER II

TOXIC EFFECTS OF SILVER NANOPARTICLES ON PLURIPOTENCY AND STEM CELL FATE IN MOUSE EMBRYONIC STEM CELLS

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ABSTRACT

Silver nanoparticles (Ag Np's) have an interesting surface chemistry and unique plasmonic properties. Ag Np toxicity studies have been limited to date which needs to be critically addressed due to its wide applications. Mouse embryonic stem (MES) cells have very stringent and tightly regulated mechanisms to circumvent DNA damage and stress response. We used 10 nm coated (polysaccharide) and uncoated Ag Np's to test its toxic effects on MES cells. MES cells and embryoid bodies (EB's) were treated with two concentrations of Ag Np's: 5 ug/ml and 50 ug/ml and exposed for 24, 48 and 72 hours. Increased ROS production led to the loss of mitochondrial membrane potential and induced cell death. Alkaline phosphatase (AP) and flow cytometry analysis revealed Ag Np's treated MES cells altered stem cell renewal capacity and cell cycle phases. Molecular analysis indicated pluripotency factors Oct4 and Nanog undergo ubiquitination and dephosphorylation post-translational modifications in Ag Np treated MES cells

thereby altering gene expression. Differentiation of EB's into all the three embryonic germ layers with specific growth factors were also inhibited after Ag Np exposure. Our results suggest that Ag Np's effect MES cell self renewal, pluripotency and differentiation and serves as a perfect model system for studying toxicity induced by engineered Ag Np's.

1. INTRODUCTION

Ag Np's are widely used in a variety of applications ranging from consumer products like socks, medical dressing, computer chips and it is also shown to have antimicrobial, anti bacterial activity and wound healing properties (Ahamed, Alsalhi, & Siddiqui, 2010; Mailander & Landfester, 2009). Since it is easy to synthesize in large quantities, Ag Np's are the most used engineered nanoparticles for commercialization and humans are constantly exposed by them through ingestion, inhalation and skin penetration (Mailander & Landfester, 2009; Park et al., 2011). Recently, studies have showed Ag Np toxicity using different model systems. Due to continuous exposure to Ag Np's, they have shown to accumulate in organs like liver, lungs and brain in Rats and Mice, cause genetic abnormalities and malfunctions during development in *Drosophila*, mice and Zebrafish (Ahamed et al., 2010; Ahamed et al., 2010; Carlson et al., 2008). Exposure to Ag Np's present in the environment accumulates in the liver and brain which increases stress responses in alveolar macrophages, lung epithelial cells and a decrease in dopamine secretion in neuro endocrine cells and additional accumulation in the lungs and kidneys (Ahamed, Akhtar, Raja et al., 2011; Ahamed, Akhtar, Siddiqui et al., 2011). Also, EGF

signaling was disrupted along with increased ROS production, decreased Akt and Erk phosphorylation, cell migration and receptor expression when human epithelial cells were exposed to 10 nm Ag NP's. Small sized nanoparticles are more toxic to mammalian cells than nanoparticles with a large diameter (Mailander & Landfester, 2009; Majeed Khan, Kumar, Ahamed, Alrokayan, & Alsalhi, 2011).

Our previous results demonstrate that 25 nm coated and uncoated Ag Np's induces the expression of the tumor suppressor protein p53, and DNA damage response proteins Rad51 and H2AX in MES cells (Ahamed et al., 2008). MES cells serves as a perfect model for toxicity studies since it can affect or alter the characteristics of self renewal, pluripotency and differentiation typically seen in MES cells. MES cells have evolved with stringent mechanisms to control DNA damage and stress response due to cytotoxic and genotoxic insults (Hong & Stambrook, 2004; C. Y. Lin et al., 2011). The network responsible for maintaining pluripotency in MES cells is tightly regulated and any changes in levels of protein expression of Oct4, Nanog and Sox2 can alter the fate of these cells (Loh et al., 2006; Pan & Thomson, 2007; Rodda et al., 2005). Exposure of MES cells to oxidized nanodiamonds showed altered Oct4 expression (Xing et al., 2011). Also 10 nm silica nanoparticles and 20 nm Ag Np's were shown to inhibit cardiomyocyte formation in MES cells (Tran, Ota, Jacobson, Patton, & Chan, 2007). Till date, no study has shown how Ag Np's affect the characteristics of MES cells by inducing stress response for longer time periods. We studied stress response in MES cells by ROS production, MMP activity, Annexin V (apoptosis) and PI (necrosis) staining and changes in cell/colony morphology, loss of self renewal capacity by AP staining, changes in cell cycle phases and post-translational modifications of Oct4 and Nanog. Western blotting

technique was used to examine changes in gene expression of pluripotency factors Oct4, Nanog and Sox2, and differentiation potential was characterized by spontaneous and directed (using growth factors) differentiation followed by quantification of pluripotency factors. Our results show detailed analysis of changes that occur at the cellular and molecular level induced by 10 nm coated and uncoated Ag Np's (5 and 50 µg/ml concentrations) in MES cell model system.

2. MATERIALS AND METHODS

Cell Culture and Ag Np treatment: Mouse embryonic fibroblast (MEF) cells (P1) were cultured at 37⁰C and 10 % CO₂ in Dulbecco's Modified Eagle Medium (DMEM, GIBCO Cat # 11965) supplemented with 5% penicillin-streptomycin (GIBCO Cat # 15140) and 10% Fetal Bovine Serum (FBS, USA Scientific # 98715200). MES cells were cultured on Mitomycin C (Sigma Cat # M4287) treated MEF cells and then passed onto 1% gelatin (Fisher scientific) coated dishes with a low dilution (excluding MEF cells) using DMEM supplemented with 0.5% penicillin-streptomycin, 1X non essential amino acids (NEAA, GIBCO Cat # 11140) and 1X Glutamax (GIBCO Cat # 35050) , 10% embryonic stem cell qualified FBS (USA Scientific Cat # 98375200), 0.1 µM β-mercaptoethanol (Sigma Cat # M6250) and 50 µM leukemia inhibitory factor (LIF, Millipore Cat # ESG 1107). Both MES cells and MEF cells were passaged using 0.25% trypsin (GIBCO Cat # 25200). Ag Np's (coated and uncoated, 10 nm in diameter) were diluted in MES cell media, sonicated at room temperature for 30 minutes at 40 W to prevent Ag Np from agglomeration. Specific concentrations of Ag Np's (5 and 50 ug/ml) were added to MES

cells (60% confluency) immediately after trypsinization into 6 well gelatin coated dishes and images were captured and cells were harvested at 24, 48 and 72 hours for further downstream analysis.

Ag Np's characterization: Hitachi H-7600 tungsten tip instrument was used to obtain TEM images at 120 kV. Suspensions of coated and uncoated Ag Np's were deposited onto copper grids coated with carbon film. AMT software and point to point method was used for was used for analyzing Ag Np's.

Alkaline Phosphatase Staining: After Ag Np treatment, MES cells were stained with Alkaline Phosphatase (Vector Labs, Cat # SK 5100) and compared to control cells. The substrate working solution was made in 100 mM Tris-HCl, pH 8.2-8.5 according to the manufacturer's protocol and the cells were incubated with the stain for 30 minutes at room temperature in the dark. The cells were then washed with PBS and images were taken using compound microscope and Micron software.

Reactive Oxygen Species Assay: Treated and untreated MES cells were incubated with 5 μ M CellROX Deep Red Reagent (GIBCO, Cat # C10422) for 30 minutes in the dark at 37⁰C and 10 % CO₂ in MES cell culture media and washed 3X in PBS (pH 7.2-7.6). Images were taken using a confocal microscope (Cy5 channel) and Fluoview image acquisition and analysis software.

Mitochondrial Membrane Potential Assay: Loss of Mitochondrial membrane potential was detected using JC-1 stain (Invitrogen, Cat # T3168). A final concentration of 10 µg/ml was used directly to treated and untreated MES cells in culture, incubated for 30 minutes at 37⁰C and 10 % CO₂ in the dark. JC-1 monomers and J aggregates were excited simultaneously at 488 nm. Emission filters for JC-1 monomer and J aggregates detection was set at 530 +/- 15 nm and 590 +/- 17.5 nm respectively. Images were taken using a confocal microscope and Fluoview image acquisition and analysis software.

Apoptosis Assay: Annexin V and Propidium iodide (Biovision, Cat # K101) was used to detect apoptotic and necrotic MES cells treated with Ag Np's respectively. Media was aspirated and incubated for 5 minutes with 1X binding buffer containing 5 µl of Annexin V-FITC and 5 µl of PI followed by fixation in 2% formaldehyde for 1 minute. Images were taken using a laser scanning confocal microscope and acquired using Fluoview software. Apoptotic and necrotic cells were detected using a 488 nm and a 540 nm excitation laser respectively.

Western Blotting: Harvested cell pellets of control and treated MES cells were lysed with 100µl RIPA buffer (Tris-HCl-pH 7.4:50mM, NP-40:1%, Sodium deoxcholate: 0.25%, NaCl: 150mM, EDTA: 1mM). The mix was centrifuged at 1000g for 1 minute and 100µl of 2X loading dye was added to 100 µl of cell lysate. The samples were then boiled at 100⁰C for 10 minutes. A sodium dodecyl sulfate polyacrylamide gel electrophoresis (15%, SDS-PAGE) was performed to separate the proteins with respect to their charge and molecular weight, and then transferred to a poly vinylidene di fluoride (PVDF,

Millipore) membrane. The membrane was blocked with 3% non fat dry milk to prevent non specific binding of primary antibodies. 2 μ g of oct4 (Santa Cruz, Cat # SC-5279), Nanog (Bethyl scientific, Cat # A300-397A), and Sox2 (R & D Systems, Cat # MAB 2018) antibodies diluted in 3% milk was added and incubated overnight at 4⁰C with shaking, washed with 1X Tris Borate Saline-Tween-20 buffer (TBST) 2 times for 10 minutes each. The respective secondary antibody was added (Santa Cruz, Cat # SC-2004 and SC-2005), incubated for 1 hour at room temperature in 3% milk, washed with TBST (4 times for 15 minutes each) followed by chemiluminescent detection (Amersham Biosciences, Cat # RPN 2106). The membrane was developed using an X-ray film (VWR, Cat # 8689358) and the protein bands were quantified using β -actin as a loading control.

Differentiation Assay: For spontaneous differentiation, MES cells were plated on 0.1% gelatin coated dishes in MES culture medium without LIF and antibiotics and the appropriate concentrations of Ag Np's were added. 7 day old EB's were collected and plated onto 0.1% gelatin coated 24 well plates and cultured in MES cell culture medium without LIF. β -NGF (R and D Systems, Cat # 256-GF) and HGF (R and D Systems, Cat # 294-HG) was added for ectoderm differentiation, all trans retinoic acid (Sigma, Cat # R2625), BMP-4 (R and D Systems, Cat # 314-BP), EGF (R and D Systems, Cat # 236-EG) and bFGF (Invitrogen, Cat # 13256-029 was added for mesoderm differentiation and Activin-A (R and D Systems, Cat # 338-AC) and TGF- β (R and D Systems, Cat # 240-B) was added for endoderm differentiation (Schuldiner, Yanuka, Itskovitz-Eldor,

Melton, & Benvenisty, 2000). MES cells and EB's were exposed to 24, 48 and 72 hrs with Ag Np's. Images were taken using an inverted phase contrast microscope.

Flow Cytometry: MES cells were treated for 24, 48 and 72 hrs with 5 and 50 µg/ml coated and uncoated Ag Np's. EZ-BrdU cell proliferation kit (Phoenix Flow Systems, Cat # AC1101) was used to determine changes in cell cycle phases in control and treated MES cells. Ag Np treated MES cells were treated with BrdU Photolyte for 24 hours prior to every time point and the procedure was followed according to the manufacturer's protocol. The centrifugation steps were at 300 g unless otherwise mentioned. After BrdU treatment, the cells were harvested using 0.25 % trypsin and centrifuged for 5 minutes. The supernatant was aspirated and the pellet was resuspended with wash buffer gently, centrifuged, discarded the supernatant and gently resuspended in 70 % (v/v) ice cold ethanol (cell density was adjusted to $1-2 \times 10^6$ cells/ml) and stored at least overnight or until ready to use at -20°C . The cells were then resuspended in wash buffer, centrifuged and the supernatant aspirated. The wash process was repeated twice and 1 ml of denaturation buffer was added to the pellet, resuspended and incubated for 30 minutes at room temperature followed by centrifugation for 10 minutes at 400 g. Immediately after aspirating the supernatant, 1 ml of neutralization buffer was added and resuspended, centrifuged at 400 g for 10 minutes. The pellet was washed with 2 ml of rinse buffer, centrifuged and the supernatant was removed. Appropriate amount of fluorescein-PRB1 (5 µl of anti BrdU and 95 µl of rinse buffer for each assay) was added to the cell pellet, resuspended and incubated for 60 minutes in the dark at room temperature. 500 µl of PI/RNase A solution was added and further incubated for 30 minutes in the dark at room

temperature. The cells were analyzed within 3 hours of staining using BD FACSCalibur. The cells were excited at 488 nm and appropriate filters were used to capture Brdu-FITC and PI positive signals in separate channels. Data analysis was performed using ModFit LT 3.1 and Cell Quest Pro software.

Calf Intestinal Phosphatase Treatment: Cell lysate was lysed with RIPA buffer and appropriate amount of Calf Intestinal Phosphatase (New England Biolabs, Cat #) according to manufacturer's protocol was added to the lysate and incubated for 1 hour at 37⁰C followed by addition of 2X laemmli buffer and western blotting probed with Nanog antibody. Cell lysate not treated with phosphatase acted as control.

3. RESULTS

3.1 Ag Np's characterization using Transmission Electron Microscope

TEM analysis showed that both coated and uncoated Ag Np's were not agglomerated and were spherical in shape (Figure 2.1a). A total of 400 particles were characterized for their mean size and shape. Coated Ag Np's had a mean and SD of 12.06 nm \pm 4.6 nm and Uncoated Ag Np's had a mean and SD of 12.21 nm \pm 5.3 nm (Figure 2.1b).

3.2 Ag Np's altered cell morphology and colony formation in MES cells

Polysaccharide coated Ag Np's and non functionalized uncoated Ag Np's were used in this study. MES cells were exposed to 5 and 50 μ g/ml concentrations of both Ag Np's for

24, 48 and 72 hrs and phase contrast images using an inverted microscope (Nikon TS 1000) were taken for each concentration and time point. Within 24 hrs after treatment with both coated and uncoated Ag Np's at 5 and 50 µg/ml, many cells started to detach from the colonies and became single floating cells. The colony morphology was irregular with rough edges which is atypical for normal MES cells (Figure 2.2). Treatment with 5 and 50 µg/ml uncoated Ag Np's had increased single cell population when compared to coated Ag Np's at 24 hrs. By 72 hrs, majority of the cells were single and detached in all colonies.

3.3 Ag Np's increased ROS production and loss of mitochondrial membrane potential (MMP) and decreased AP expression

ROS, an indicator of cells under stress was also increased by 24 hrs and was seen at higher levels till 72 hrs for both types of particles and concentrations when compared to control cells (Figure 2.4a and b). JC-1 (5, 5', 6, 6'-tetrachlor-1, 1', 3, 3'- tetraethyl-benzamidazolocarboyanin iodide) is a dye used to detect MMP. JC-1 aggregates in normal mitochondria and fluoresces red and stays as monomers in mitochondria with collapsed MMP and fluoresces green. After staining with JC-1, both coated and uncoated Ag Np treated MES cells loose MMP by 48 hrs (Figure 2.5a and b) and exhibits a leaky membrane which may be a result of increased ROS production. AP expression is found exclusively in ES cells and acts as a marker for undifferentiated pluripotent ES cells (Zhao & Xu, 2005; Zhu, Chang, Dai, & Hong, 2007). When MES cells were exposed to Ag Np's, loss of stemness was observed wherein detached cells completely lost AP

activity within 24 hrs when treated with coated and uncoated Ag Np's at 5 and 50 $\mu\text{g/ml}$ (Figure 2.3) MES cells after 48 and 72 hrs of treatment showed an increase in single cell population expressing decreased or no AP activity. The very few cells remaining produced clumps which were still positive for AP. Our results show that MES cells treated with coated and uncoated Ag Np's (5 and 50 $\mu\text{g/ml}$) show increased ROS production and loss of MMP which are typical of cells under stressful conditions and also loose AP expression.

3.4 Annexin V expression and PI staining was increased in a time and dose dependent manner

We studied apoptosis response by using an apoptotic marker, Annexin V. After treatment, MES cell colonies showed a gradual increase in early apoptosis in a time and concentration dependent manner and high percentage of cells undergoing apoptosis were positive for Annexin V. At the same time, PI which marks necrotic cells exhibiting leaky cell membranes increased consistently with time and dosage (Figure 2.6a and b).

Uncoated 24 hr treatment showed a higher percentage of cells positive for apoptosis compared to coated 24 hr treatment. Also for all time points, uncoated Ag Np treated cells underwent an increased apoptotic rate than coated Ag Np treated cells. Stress signals induced by Ag Np's in MES cells by ROS induction and MMP loss can activate caspase activity and cytochrome release which can further activate DNA damage and apoptosis (Ahamed et al., 2008; Ahamed et al., 2010; Ahamed, Akhtar, Raja et al., 2011). Our

results confirm that uncoated Ag Np's are comparatively more toxic to MES cells than coated Ag Np's and both particles induced apoptosis.

3.5 Pluripotency factors undergo post-translational modifications after Ag Np exposure

Western blotting results show that Oct4 proteins in Ag Np treated cells undergoes ubiquitination (Figure 2.7), a typical post-translational modification which marks the protein for degradation. Also Nanog undergoes dephosphorylation (Figure 2.8) after Ag Np treatment which destabilizes and marks the protein for degradation. To further confirm Nanog dephosphorylation, we incubated the 72 hr uncoated Ag Np treated cells with calf intestinal phosphatases to see an increase in protein content in the lower band (dephosphorylated) (Figure 2.8, lower panel). After 72 hrs post treatment, increased ubiquitination of Oct4 and dephosphorylation of Nanog suggests loss of pluripotency in MES cells and further committing these cells to differentiate. Oct4 and Nanog levels are tightly regulated within a very narrow window range and slight alterations in expression of these pluripotency factors can destine those cells towards a trophectodermal (increased Oct4 expression) or an embryonic endoderm (decreased Oct4 expression) lineage (Chambers & Smith, 2004; Loh et al., 2006; Pan & Thomson, 2007). Nanog on the other hand orchestrate with Oct4 and Sox2 to maintain the pluripotency interacting network in ES cells (Pan & Thomson, 2007). Repression of Nanog by increase in p53 stress response signaling pathway will result in spontaneous differentiation of ES cells (C. Y. Lin et al., 2011; Q. Lin, Donahue, & Ruley, 2006; Rodda et al., 2005). Till date, our study is the

first of its kind to show loss of pluripotency and stemness in MES cells induced by Ag Np's.

3.6 MES cells show altered cell cycle phases induced by Ag Np's

We show that Ag Np treated MES cells have drastically altered their cell cycle phases (Figures 2.9a and 2.9b). Coated 24 hr treated cells (5 $\mu\text{g/ml}$) had no S phase activity and a higher percentage of cells in G1 (45.1%) and G2 (54.9%) compared to the control (G1 = 23.19%, G2 = 17.08%). Similar pattern was seen in uncoated 24 hr treated cells (50 $\mu\text{g/ml}$) with ~40% of the cells in G1 and ~60% in G2. In 48 and 72 hr time points, these cells showed an unusual percentage of cells in S phase {(Coated 5-48 = ~80%, 5-72 = ~71%), (Uncoated 50-48 = ~83%, 50-72 = ~97%)}. Due to increased S phase, MES cells double the mutated DNA content and activate the apoptotic cascade and eliminate these cells. Uncoated 24 hr treated cells (5 $\mu\text{g/ml}$) followed a similar trend till 48 hrs (S = ~83%) and by 72 hrs, majority of the cell population was in G2 (42%) (Figure 2.9b). This corresponds well with our previous results where there is increased apoptosis in these populations as those cells have multiplied their cell death signaling pathway prone to be eliminated thereby avoiding a major threat during embryonic development. Conversely, coated 50-24 hr treatment showed increased S phase (75%) triggering an alternative DNA damage pathway (Figure 2.9b). MES cells divide rapidly (10-12 hrs), lack a G1/S checkpoint as observed in other somatic cells and typically have a longer S phase (60-70%) and a shorter G1 and G2 phases of the cell cycle (Aarts & te Riele, 2010; Kang et al., 2009; Momcilovic, Navara, & Schatten, 2011). DNA damage and increased genetic

mutations due to genotoxic insults can hamper the phases of cell cycle and affect stem cell self renewal. MES cells accumulating DNA damage will now progress into the S phase of the cell cycle (due to lack of G1 phase) thereby increasing DNA content and exacerbating the extent of DNA damage which eliminates these cells through apoptosis (Stambrook, 2007; Teyssier, Bay, Dionet, & Verrelle, 1999). Together these results show altered cell cycle phases when MES cells were treated with coated and uncoated Ag Np's thereby showing altered self renewal capacity.

3.7 Spontaneous and directed differentiation into all 3 germ layers is inhibited by Ag Np's

First, we studied whether Ag Np's induced spontaneous differentiation in MES cells by activating p53 which suppresses Nanog promoting differentiation. We see that, coated and uncoated Ag Np's (5 and 50 $\mu\text{g/ml}$) inhibited spontaneous differentiation and the cells retained a rounded morphology with irregular colony formation (Figure 2.10). MES cells triggered apoptosis with time rather than differentiation. To analyze the molecular circuitry of pluripotency during differentiation, EB's were generated (7 days old) and were allowed to differentiate into all the 3 germ layers by adding specific growth factors (Schuldiner et al., 2000). No signs of differentiation were observed even after 72 hrs of coated (2.11a) and uncoated (2.11b) Ag Np treatment for either concentrations suggesting that directed differentiation potential of EB's was inhibited due to Ag Np induced toxicity and altered gene expression. To further quantify pluripotency associated (Oct4, Nanog and Sox2) gene expression, we performed western blotting to see if the levels

were altered during the differentiation process. At day 10 (7 days after EB formation and 3 days after directed differentiation), control EB's down regulated Oct4, Nanog and Sox2 which was typical for cells undergoing differentiation (Figure 2.12). To our surprise, we noticed that 2 out of the 3 pluripotency factors, Oct4 and Nanog expression after quantification was significantly increased by 24, 48 and 72 hrs after treatment with coated (Figure 2.13a) and uncoated (Figure 2.13b) Ag Np's for both concentrations. To further investigate toxicity of Ag Np's on MES cells, we addressed whether differentiation potential of MES cells was affected, a hallmark of ES cells. Upon withdrawal of LIF, MES cells spontaneously differentiate into random lineages within 5 days. Oct4, Nanog and Sox2 (pluripotency factors) expression is down regulated and lineage commitment genes are up regulated during the differentiation process. Silica nanoparticles with a 10 nm and 30 nm diameter inhibited spontaneously contracting cardiomyocytes in D3 murine embryonic stem cells (Tejedo et al., 2010; Tran et al., 2007). Our results is the first study to show the inhibitory effects of Ag Np's on directed differentiation of EB's to all the 3 embryonic germ layers (ectoderm, endoderm and mesoderm).

4. DISCUSSION

Ag Np's exhibit high surface area and high rates of effective collision making it highly reactive to biomolecules (Carlson et al., 2008; Gopinath, Gogoi, Chattopadhyay, & Ghosh, 2008; Gopinath et al., 2010). The surface of Ag Np's can be easily functionalized or coated with polysaccharides thereby easily changing its reactive properties and coating

of drugs with Ag Np's increases the bioavailability of drugs in the blood system (Majeed Khan et al., 2011). Ag Np's have found their way into numerous applications which are in direct contact with humans (Mailander & Landfester, 2009). Recent studies have shown toxic effects of Ag Np's on mammalian and non mammalian cells by inducing cell death, DNA damage and stress response (Ahamed et al., 2010). MES cells are pluripotent cells capable of giving rise to all tissues of the body (Chambers & Smith, 2004). We show that Ag Np's induce toxicity by increasing ROS production (cell stress, due to an imbalance in antioxidant activity), Annexin V (apoptosis) expression, PI (necrosis) staining and loss of MMP in MES cells which have dynamic stress response activation signals compared to somatic cells. Prolonged exposure to Ag Np's further increased cell stress and cell death and the stress response signaling network activated to restore normal cell functions was no match for Ag Np induced toxicity. Our results correlate with other studies showing activation of programmed cell death and increased ROS activity upon nanoparticles exposure to mammalian cells (Aarts & te Riele, 2010; Ahamed, Akhtar, Siddiqui et al., 2011; Hong, Cervantes, & Stambrook, 2006). Upon differentiation, ES cells lose AP activity and hence their pluripotency (Zhu et al., 2007). Multi walled carbon nanotubes show decreased AP activity by 24 hrs (Xing et al., 2011; Zhu et al., 2007). We show that MES cells lost AP activity after Ag Np treatment. Coated and uncoated Ag Np's induced post-translational modifications of the master regulator protein, Oct4 and Nanog, both involved in pluripotency maintenance in ES cells. Oct4 showed typical patterns of ubiquitination and Nanog showed dephosphorylation, both modifications targeting for protein degradation. Dephosphorylation of Nanog can destabilize the protein under stress conditions. We also see a slight increase in Oct4 and

Nanog expression after Ag Np treatment suggesting pluripotency factors might also have a role in activating stress response signals crucial for MES cell survival. One study has shown Oct4 to be critical for survival during stress conditions and indirectly activates Survivin through signal transducers and activators of transcription 3 (STAT3) pathway (Guo, Mantel, Hromas, & Broxmeyer, 2008). Sox2 also showed increased expression along with Oct4 and Nanog (data not shown). These results suggest that the pluripotency interacting network works together during genotoxic insults in MES cells. To our knowledge, this is the first study to show increased expression of Oct4, Nanog and Sox2 after Ag Np treatment in MES cells. Further, cells in different phases of cell cycle were altered when compared to control MES cells due to the interaction with Ag Np's suggesting these cells lose self renewal capacity and pluripotency. Finally, differentiation potential of EB's into all 3 germ layers and spontaneous differentiation of MES cells in culture is impaired due to Ag Np's. This can be a major concern for adult stem cells exposed to Ag Np's for long time periods in the body. The regenerative capacity of stem cells will be lost and no replacement of damaged tissue due to injury or normal wear and tear of the body will be possible.

Together, our study demonstrates that loss of self renewal capacity, decreased proliferation, altered expression of pluripotency governing factors and inhibition of differentiation are all induced by both coated and uncoated Ag Np's at 5 and 50 $\mu\text{g/ml}$ concentrations in MES cells. Therefore Ag Np's have to be assessed for toxicity in mammalian cells before commercialization and we show that MES cells can serve as an excellent model system to thoroughly characterize toxicity in the cellular and molecular levels.

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Figure 2.1a Ag Np's characterization. Ag Np suspensions were dried on carbon film coated copper grids. AMT camera was used for image capture. Images were taken at 120,000 X magnification and 120 kV. Coated Ag Np's - top and Uncoated Ag Np's - bottom. Scale bar 20 nm.

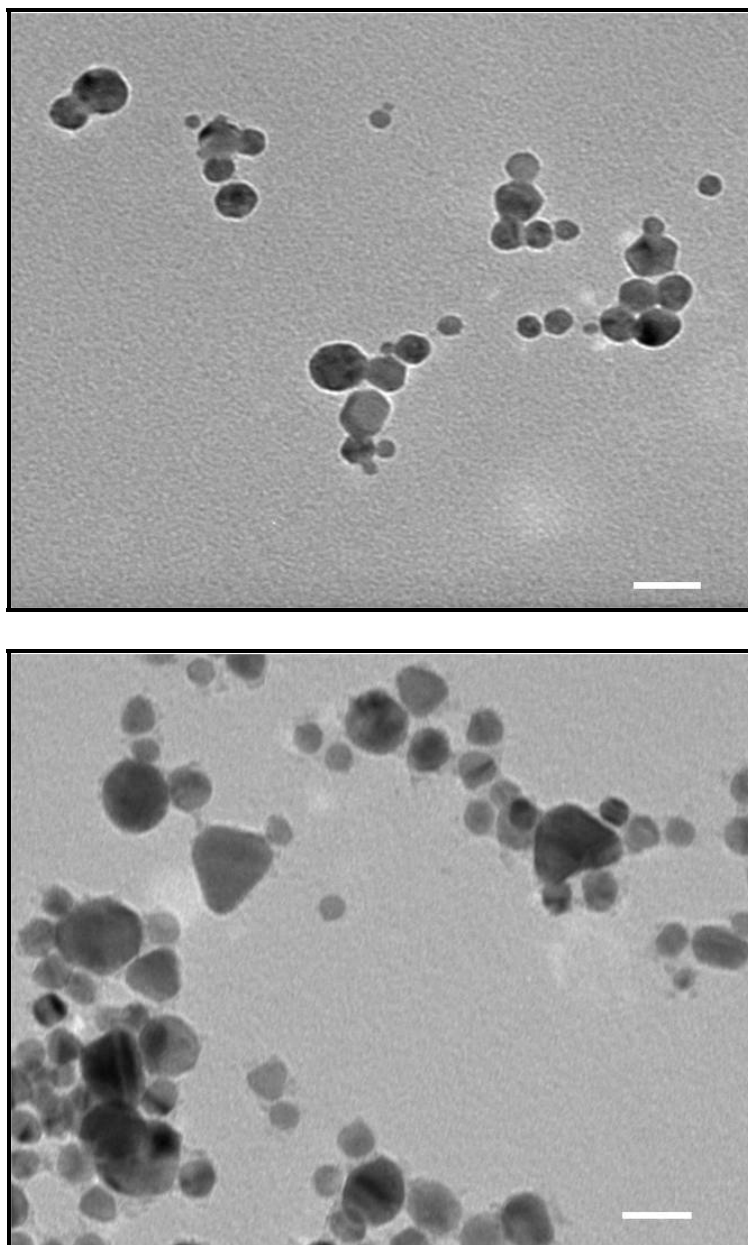


Figure 2.1b Size distribution of Ag Np's. A total of 400 Ag Np's were measured using transmission electron microscope. AMT software was used for size measurements. Point to point method was employed for calculating mean size (Coated - 12.06 nm, Uncoated - 12.21 nm) and SD (Coated - ± 4.6 nm, Uncoated - ± 5.3 nm). Coated Ag Np's - top and Uncoated Ag Np's - bottom.

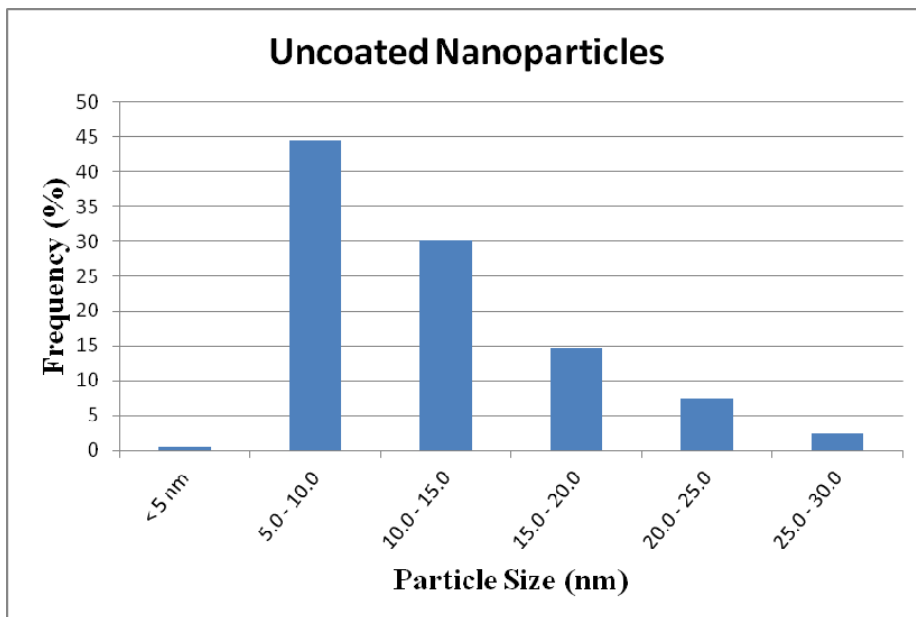
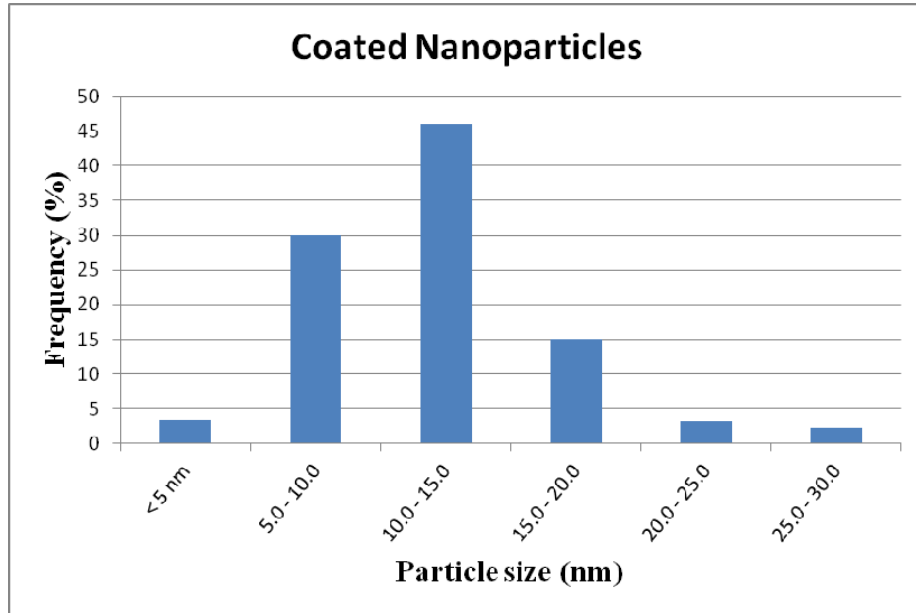
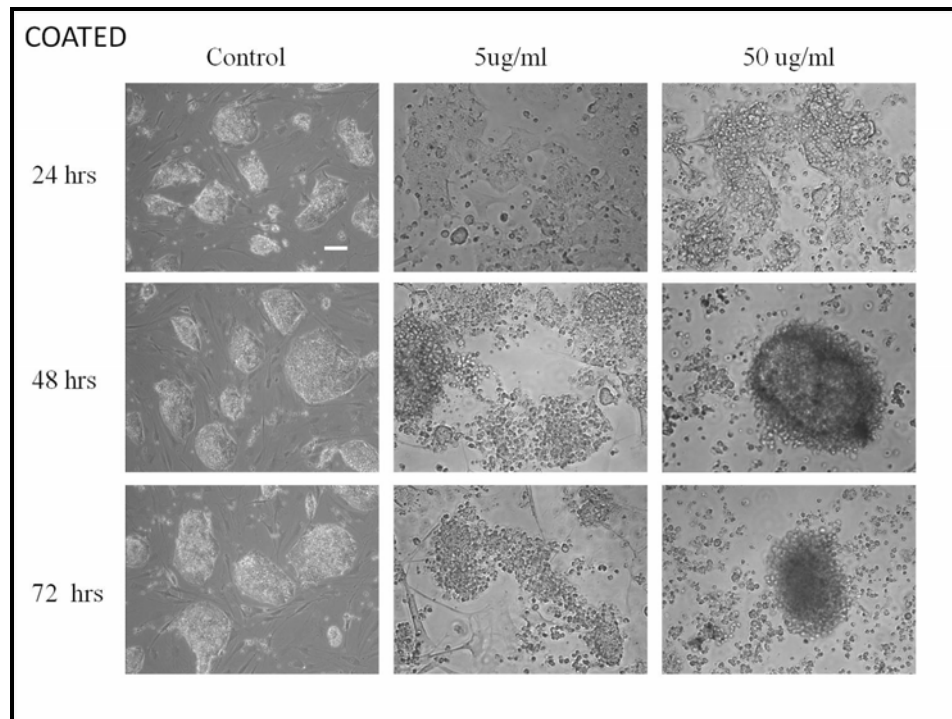


Figure 2.2: Ag Np's altered cell morphology and colony formation in MES cells. MES cells were treated with coated (top panel) and uncoated Ag Np's (bottom panel). Left column is control MES cells, centre column is 5 $\mu\text{g}/\text{ml}$ and right column is 50 $\mu\text{g}/\text{ml}$ treatments. First, second and third row in both panels represent 24 hrs, 48 hrs and 72 hrs time point respectively. The images were captured at 10 X magnification using an inverted microscope (Nikon TS 1000). Scale bar is 100 μm .



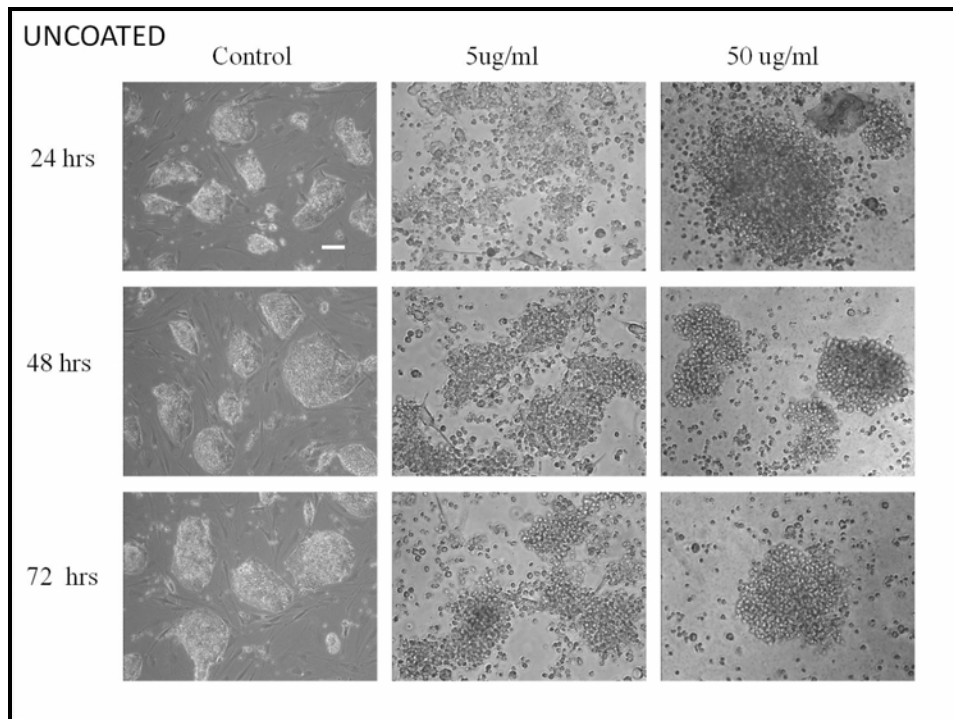
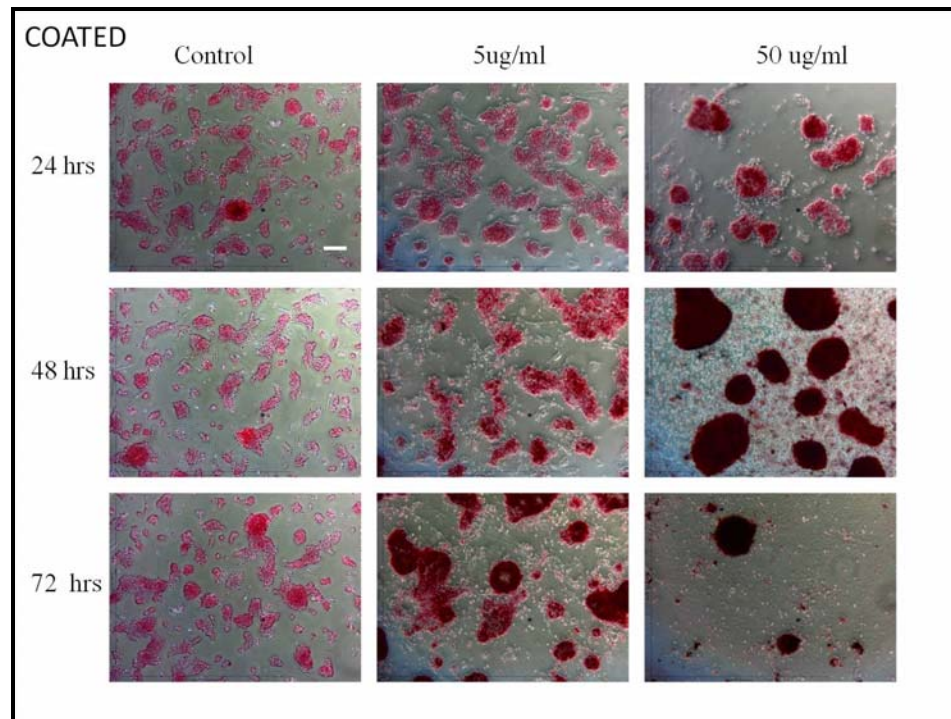


Figure 2.3: AP activity is decreased upon Ag Np treatment in MES cells. MES cells were treated with coated (top panel) and uncoated Ag Np's (bottom panel). Left column is control MES cells, centre column is 5 $\mu\text{g/ml}$ and right column is 50 $\mu\text{g/ml}$ treatments. First, second and third row in both panels represent 24 hrs, 48 hrs and 72 hrs time point respectively. The images were captured at 10 X magnification using an inverted microscope (Nikon TS 1000). Scale bar is 100 μm .



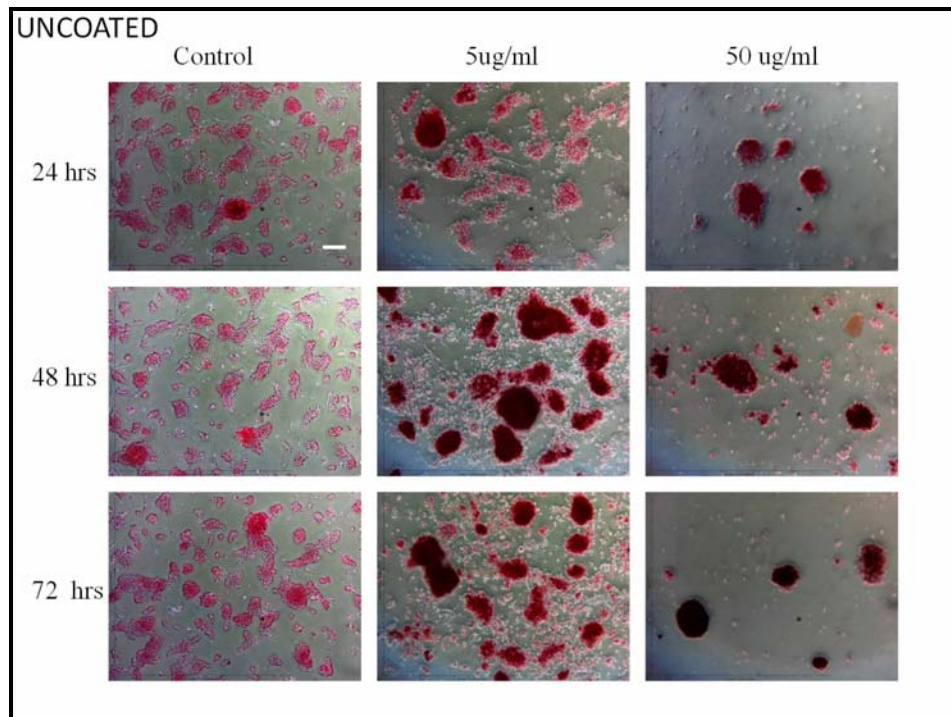
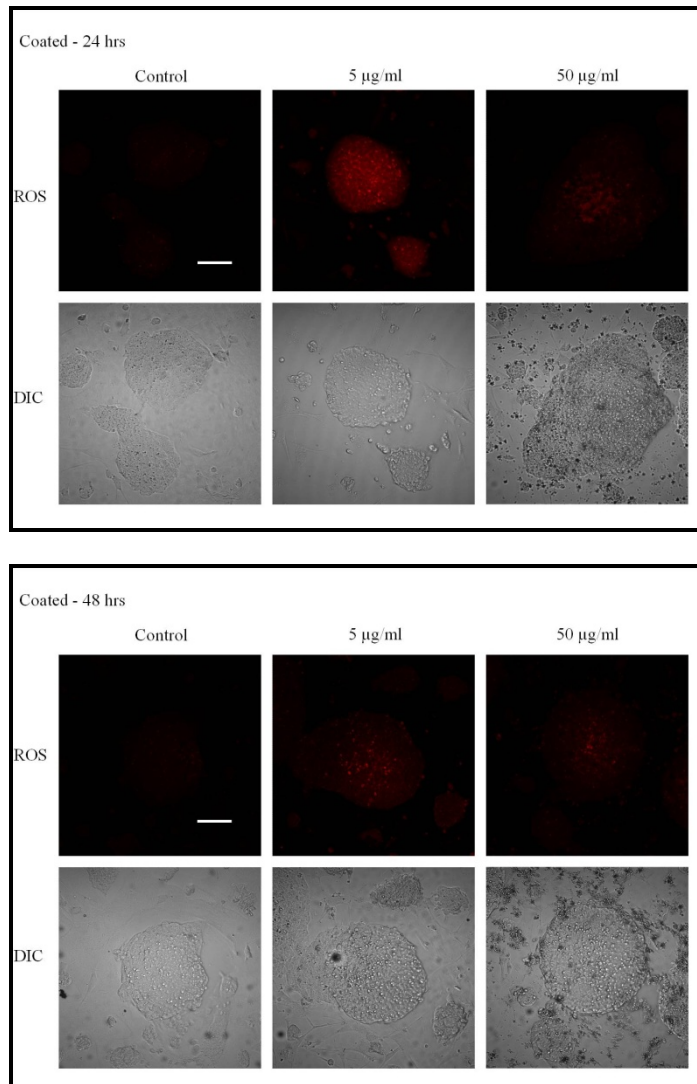


Figure 2.4a: ROS production is increased in MES cells after coated Ag Np treatment. Top, centre and lower panel represent 24 hrs, 48 hrs and 72 hrs time points respectively. Fluorescent (top row) and DIC (bottom row) images were taken for each time point. Left column is control MES cells, centre column is 5 $\mu\text{g/ml}$ and right column is 50 $\mu\text{g/ml}$ treatments. Images were captured at 20 X magnification using a confocal microscope (Olympus FV 1000). Scale bar is 100 μm .



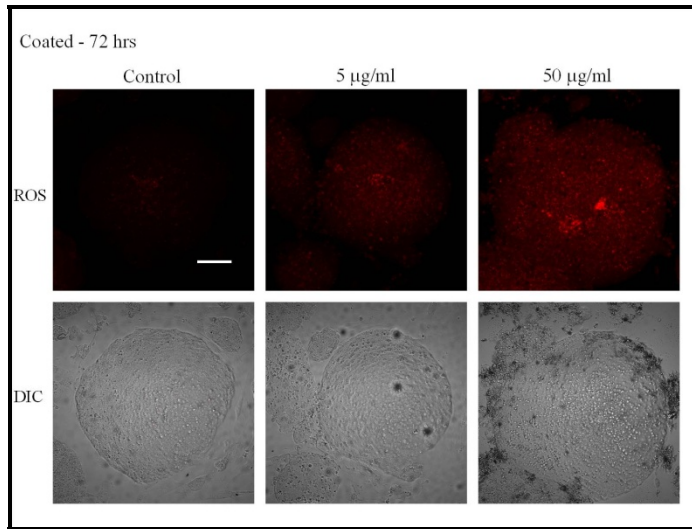
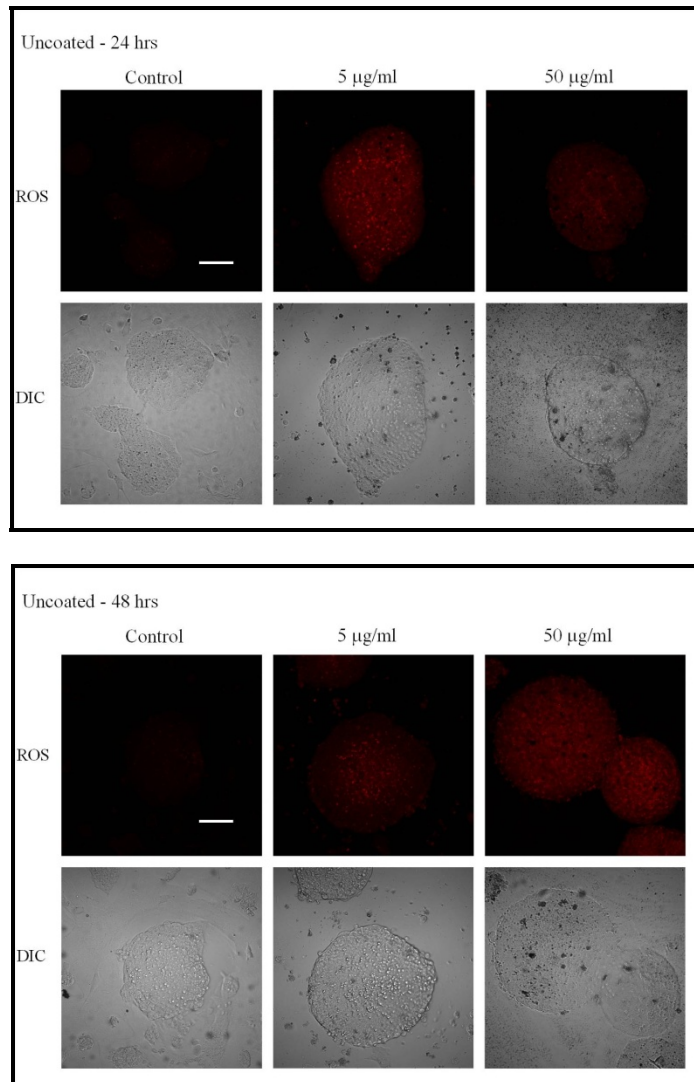


Figure 2.4b: ROS production is increased in MES cells after uncoated Ag Np treatment. Top, centre and lower panel represent 24 hrs, 48 hrs and 72 hrs time points respectively. Fluorescent (top row) and DIC (bottom row) images were taken for each time point. Left column is control MES cells, centre column is 5 $\mu\text{g/ml}$ and right column is 50 $\mu\text{g/ml}$ treatments. Images were captured at 20 X magnification using a confocal microscope (Olympus FV 1000). Scale bar is 100 μm .



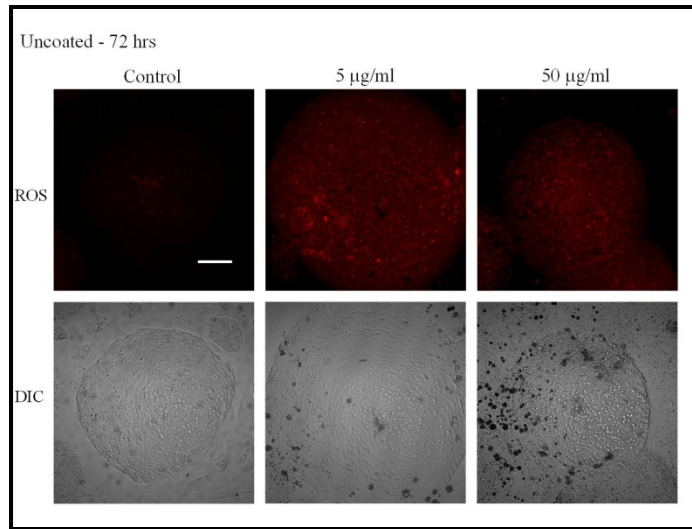
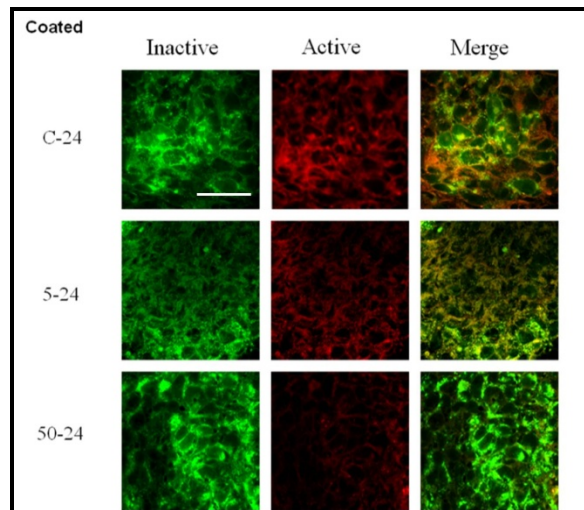


Figure 2.5a: Mitochondrial membrane potential is lost in MES cells treated with coated Ag Np's. MES cells stained with JC-1 to detect membrane potential activity. Top, centre and lower panel represent 24 hrs, 48 hrs and 72 hrs time points respectively. Top row showing control MES cells, centre row showing 5 $\mu\text{g}/\text{ml}$ and bottom row showing 50 $\mu\text{g}/\text{ml}$ treatments. Left column represents mitochondria that are inactive or lost membrane potential. Centre column represents active mitochondria with have retained membrane potential and right column represents a merged image of active (centre column) and inactive (left column) mitochondria. Images were captured at 100 X magnification using a confocal microscope (Olympus FV 1000). Excitation was at 488 nm and emission filters were set at 530 ± 15 nm (inactive) and > 590 nm (active). Scale bar is 50 μm .



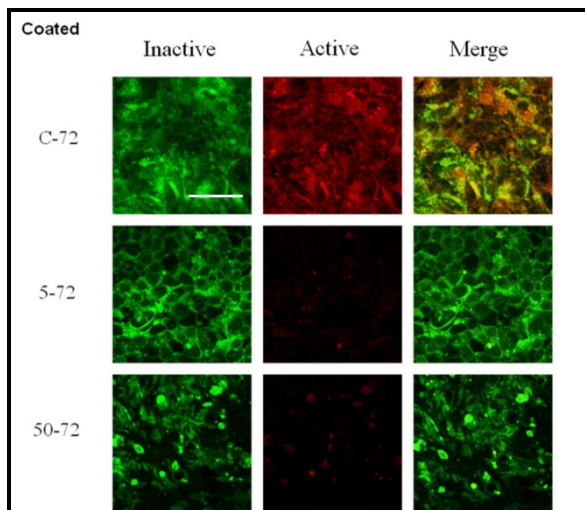
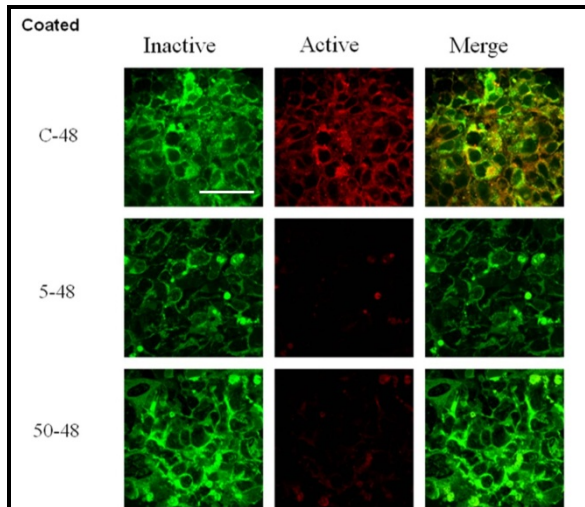
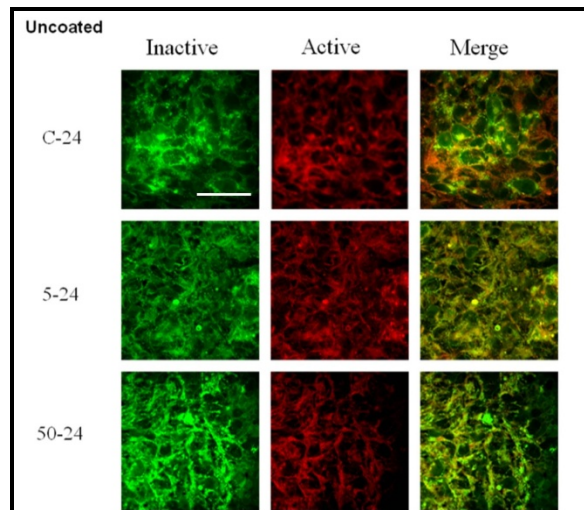


Figure 2.5b: Mitochondrial membrane potential is lost in MES cells treated with uncoated Ag Np's. MES cells stained with JC-1 to detect membrane potential activity. Top, centre and lower panel represent 24 hrs, 48 hrs and 72 hrs time points respectively. Top row showing control MES cells, centre row showing 5 $\mu\text{g/ml}$ and bottom row showing 50 $\mu\text{g/ml}$ treatments. Left column represents mitochondria that are inactive or lost membrane potential. Centre column represents active mitochondria with have retained membrane potential and right column represents a merged image of active (centre column) and inactive (left column) mitochondria. Images were captured at 100 X magnification using a confocal microscope (Olympus FV 1000). Excitation was at 488 nm and emission filters were set at 530 ± 15 nm (inactive) and > 590 nm (active). Scale bar is 50 μm .



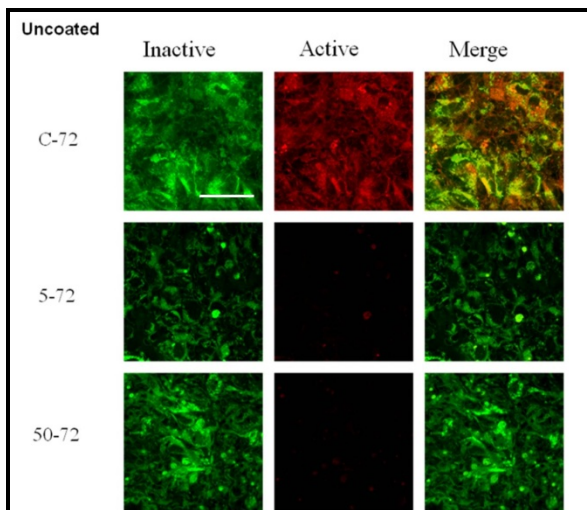
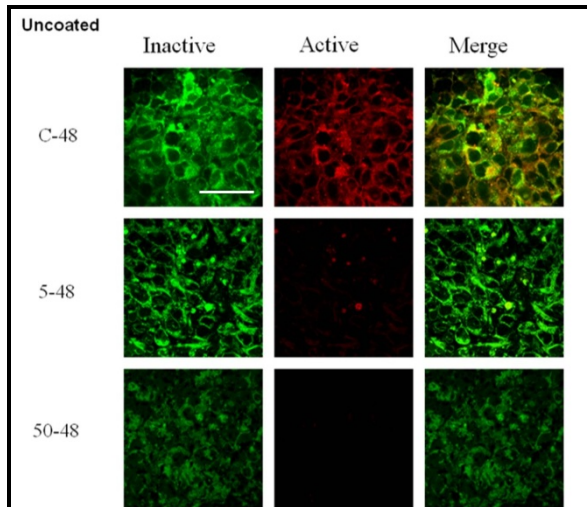
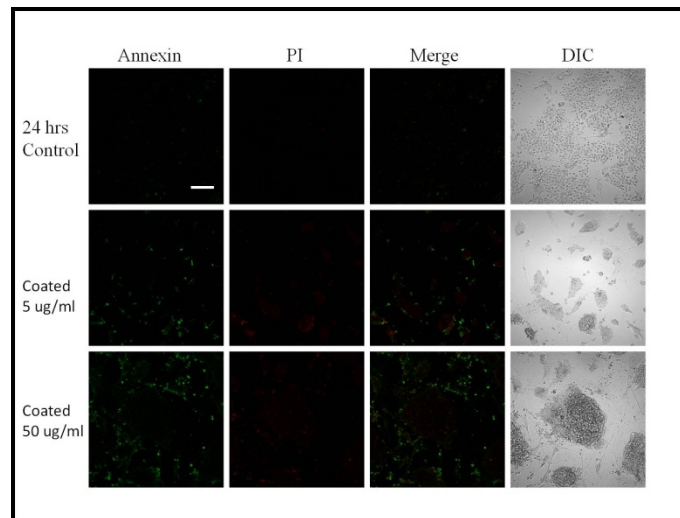


Figure 2.6a: Annexin V and PI staining (coated Ag Np's). Annexin V apoptosis marker and PI staining is increased in a time and a concentration dependent manner in MES cells treated with coated Ag Np's. Top, centre and lower panel represent 24 hrs, 48 hrs and 72 hrs time points respectively. Top row showing control MES cells, centre row showing 5 $\mu\text{g}/\text{ml}$ and bottom row showing 50 $\mu\text{g}/\text{ml}$ treatments. Left column represents Annexin V-FITC positive cells (apoptosis), centre left column represents PI staining (necrosis), centre right column represents a merged image of Annexin V and PI staining and right column represents DIC images. Annexin V-FITC signal was detected using a 488 nm excitation channel and PI signal was detected using a Cy 5 channel. Images were captured at 20 X magnification using a confocal microscope (Olympus FV 1000). Scale bar is 100 μm .



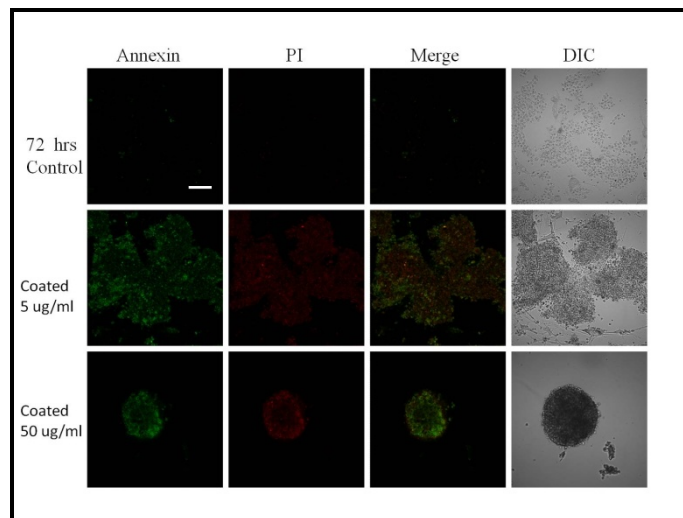
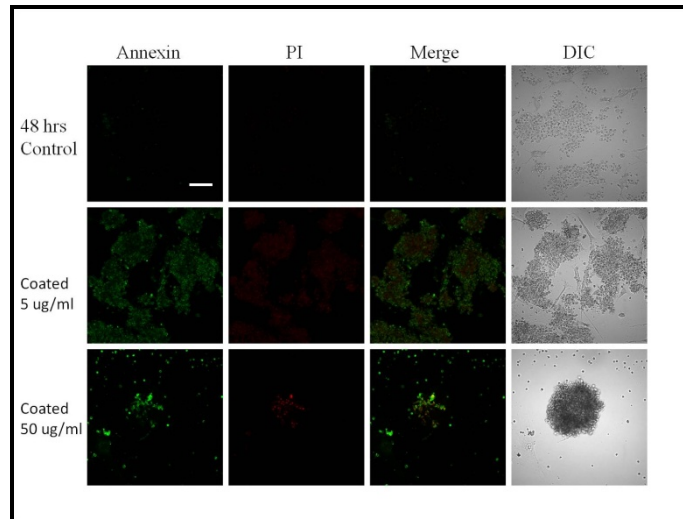
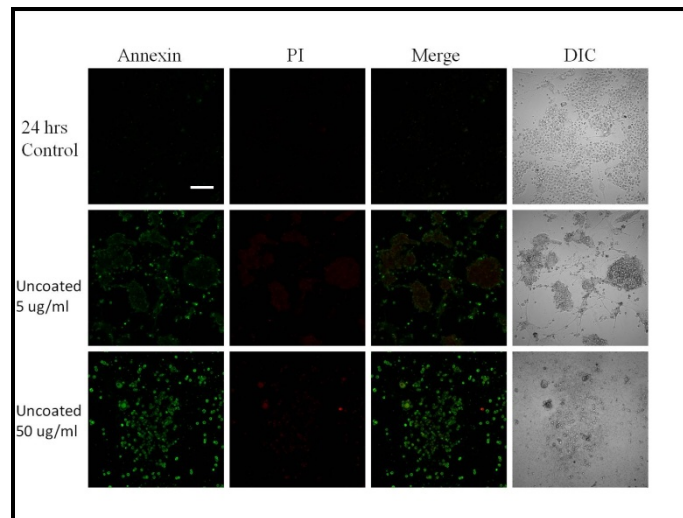


Figure 2.6b: Annexin V and PI staining (uncoated Ag Np's). Annexin V apoptosis marker and PI staining is increased in a time and a concentration dependent manner in MES cells treated with uncoated Ag Np's. Top, centre and lower panel represent 24 hrs, 48 hrs and 72 hrs time points respectively. Top row showing control MES cells, centre row showing 5 $\mu\text{g}/\text{ml}$ and bottom row showing 50 $\mu\text{g}/\text{ml}$ treatments. Left column represents Annexin V-FITC positive cells (apoptosis), centre left column represents PI staining (necrosis), centre right column represents a merged image of Annexin V and PI staining and right column represents DIC images. Annexin V-FITC signal was detected using a 488 nm excitation channel and PI signal was detected using a Cy 5 channel. Images were captured at 20 X magnification using a confocal microscope (Olympus FV 1000). Scale bar is 100 μm .



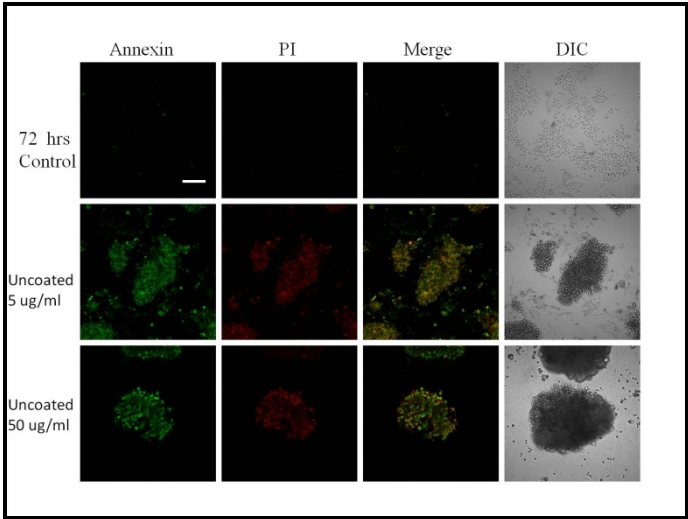
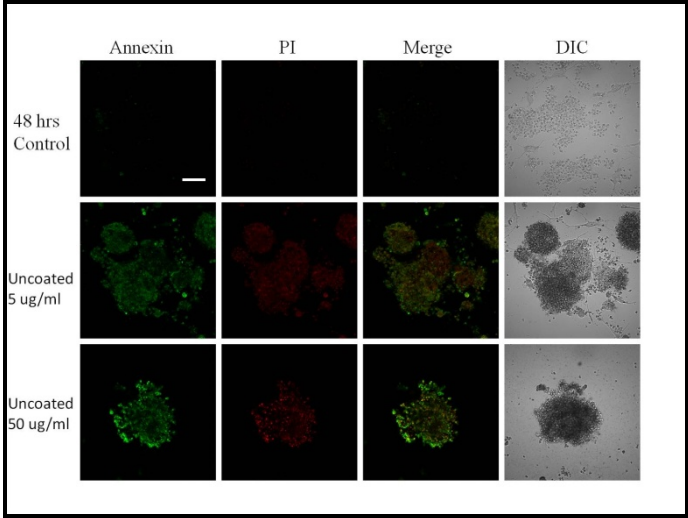


Figure 2.7: Oct4 undergoes post-translational modifications after Ag Np exposure.

Western Blot analysis of Oct4 protein expression in MES cells treated with coated (left, 5 and 50 $\mu\text{g/ml}$) and uncoated (right, 5 and 50 $\mu\text{g/ml}$) Ag Np's for 24, 48 and 72 hrs. Red box showing bands of ubiquitinated Oct4 protein. β -actin was used as a loading control.

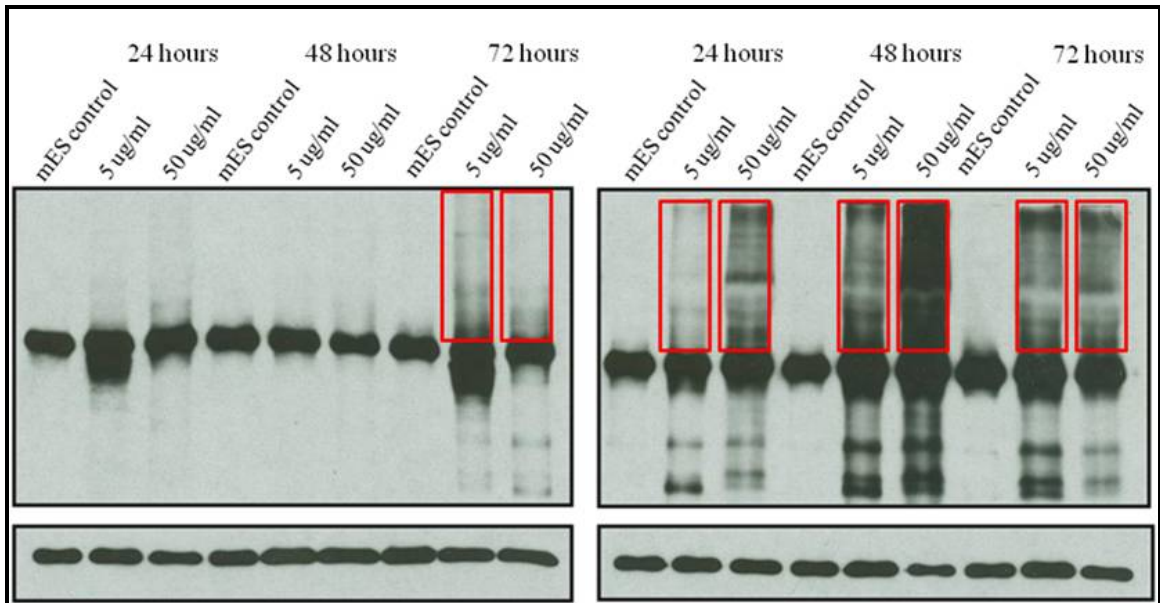


Figure 2.8: Nanog undergoes post-translational modifications after Ag Np exposure.

Western Blot analysis of Nanog protein expression (top panel) in MES cells treated with coated (left, 5 and 50 $\mu\text{g/ml}$) and uncoated (right, 5 and 50 $\mu\text{g/ml}$) Ag Np's for 24, 48 and 72 hrs. Arrow pointing to dephosphorylated Nanog protein. Lower panel showing 72 hrs time point of MES cells treated with calf intestinal phosphatases (CIP) exposed to uncoated Ag Np's. Left column - without CIP treatment, right column - after CIP treatment. Red box represents the extent of Nanog dephosphorylation. β -actin was used as a loading control.

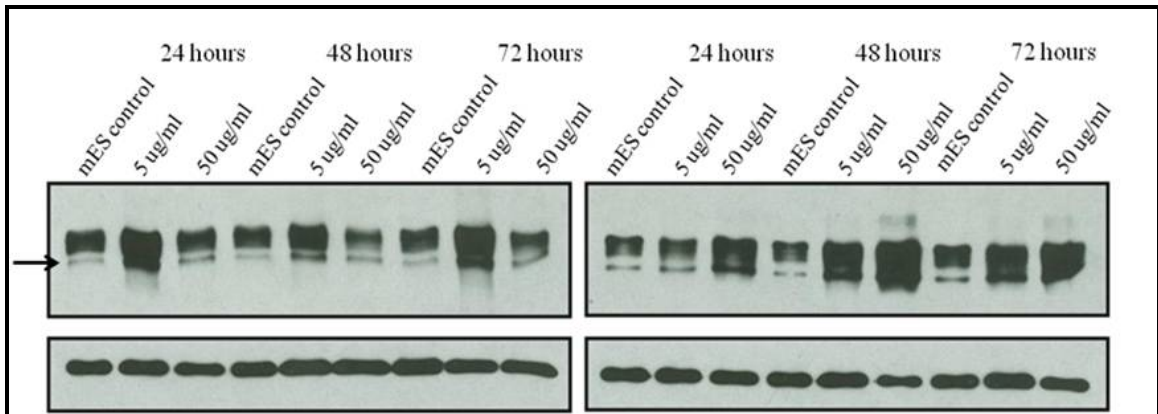
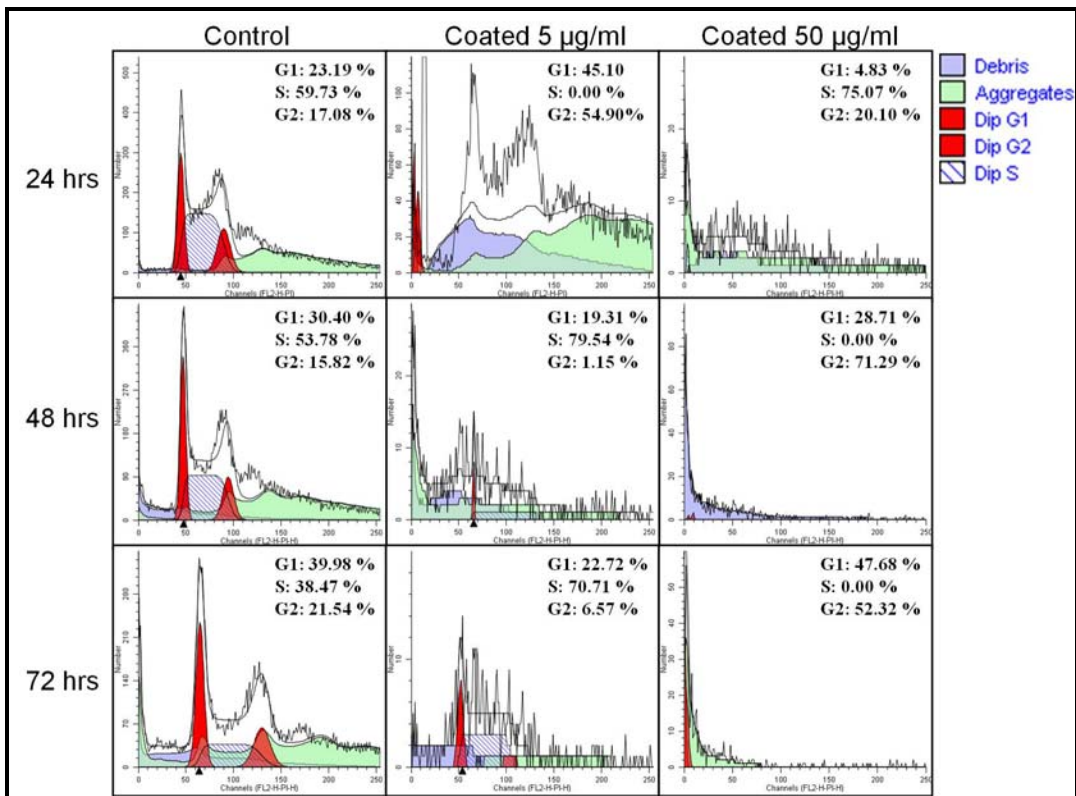


Figure 2.9a: Ag Np's influence on cell cycle phases in MES cells. Ag Np's affect self renewal capacity by altering cell cycle phases in MES cells treated with coated and uncoated Ag Np's at 5 and 50 $\mu\text{g/ml}$. Data showing flow cytometry analysis by plotting cell count versus DNA content. MES cells were treated with coated (top panel) and uncoated Ag Np's (bottom panel). Left column is control MES cells, centre column is 5 $\mu\text{g/ml}$ and right column is 50 $\mu\text{g/ml}$ treatments. First, second and third row in both panels represent 24 hrs, 48 hrs and 72 hrs time point respectively. PI intensity represents DNA content. Cells with 2N DNA content represents G1 peak and cells with 4N DNA content represents G2 peak and cells in the S phase are represented in between G1 and G2 peaks. BD FACSCalibur instrument was used for flow cytometry. Cell Quest Pro and Mod Fit LT 3.2 were used for data acquisition and analysis.



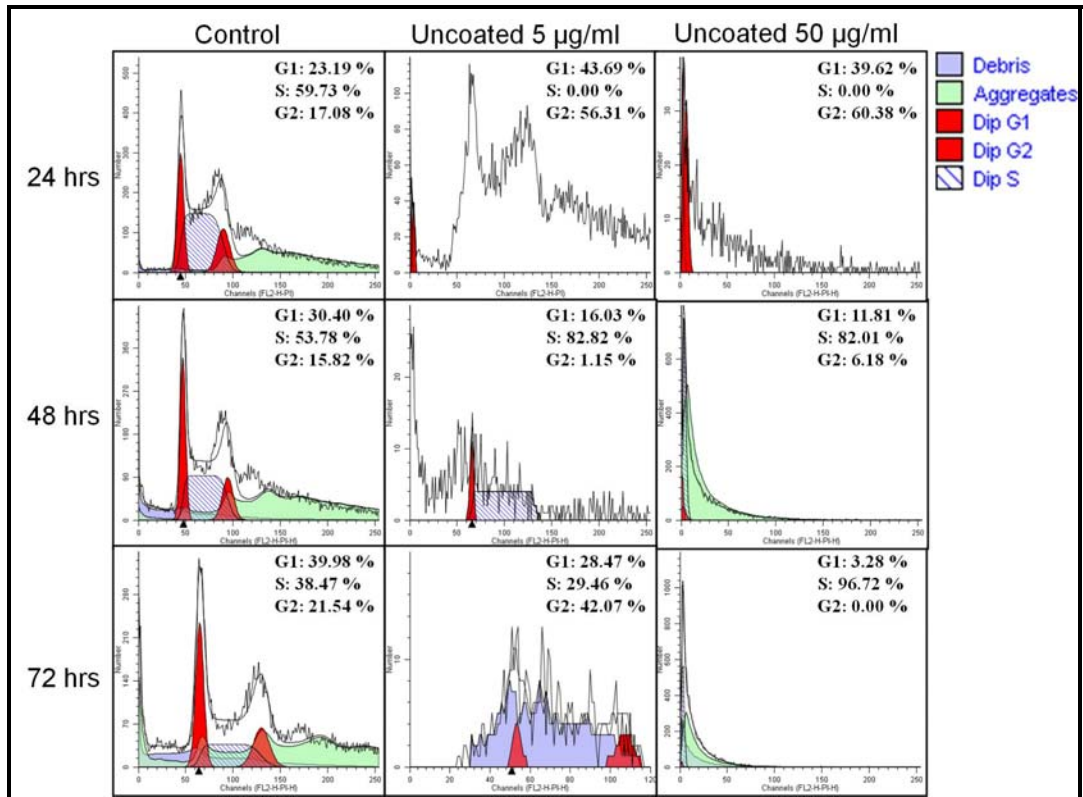
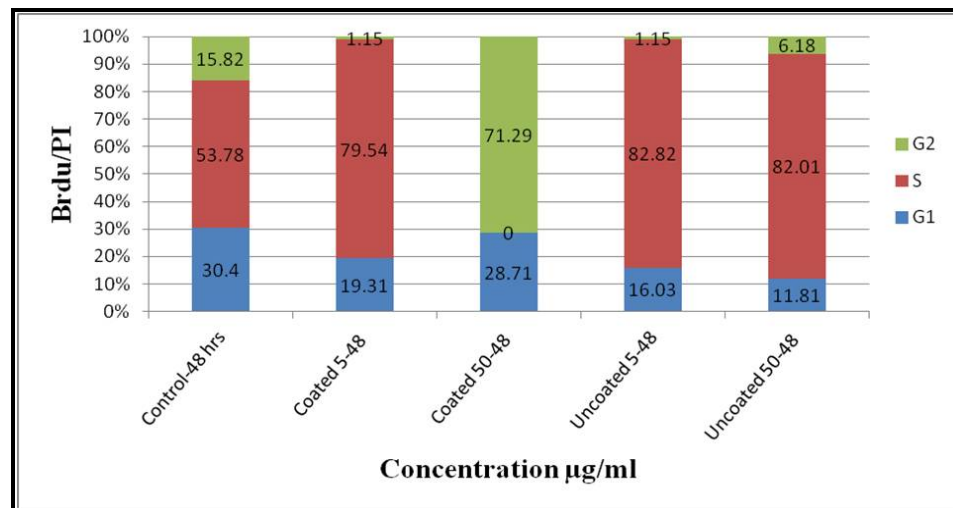
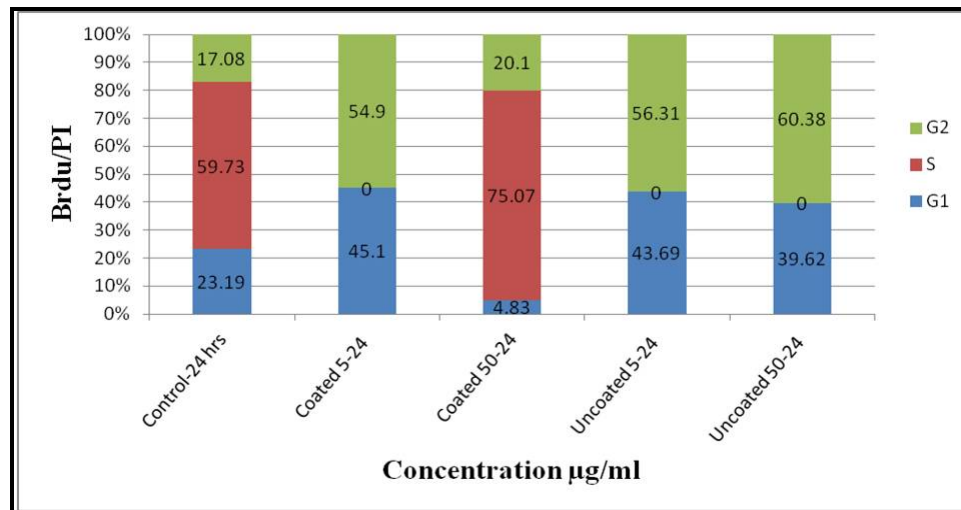


Figure 2.9b: Ag Np's inducing MES cells to be in S phase and activate apoptosis. Brdu marks proliferating cells and PI marks total DNA content. Top, centre and lower graphs represent 24 hrs, 48 hrs and 72 hrs time points respectively. Each panel contains the respective control, coated and uncoated Ag Np's (5 and 50 µg/ml) treated MES cells. Graphs were analyzed using Microsoft Excel software.



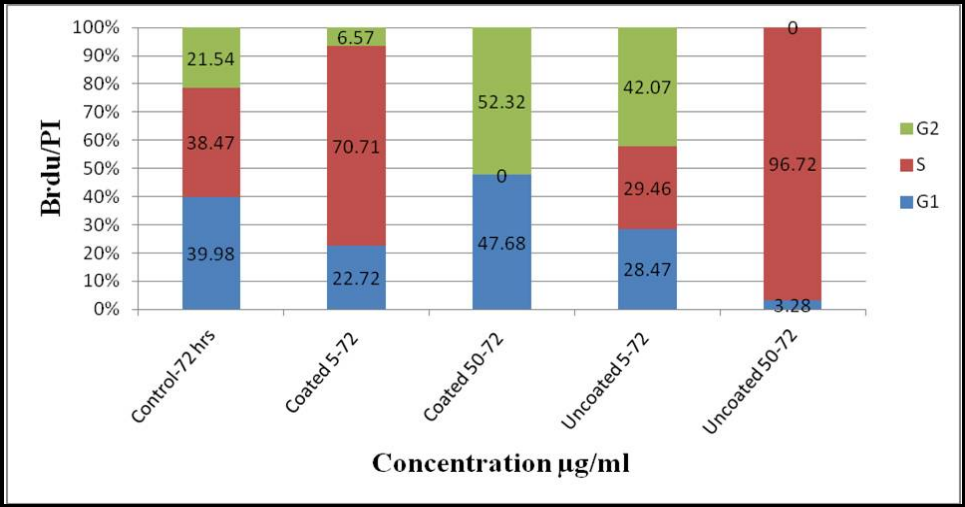
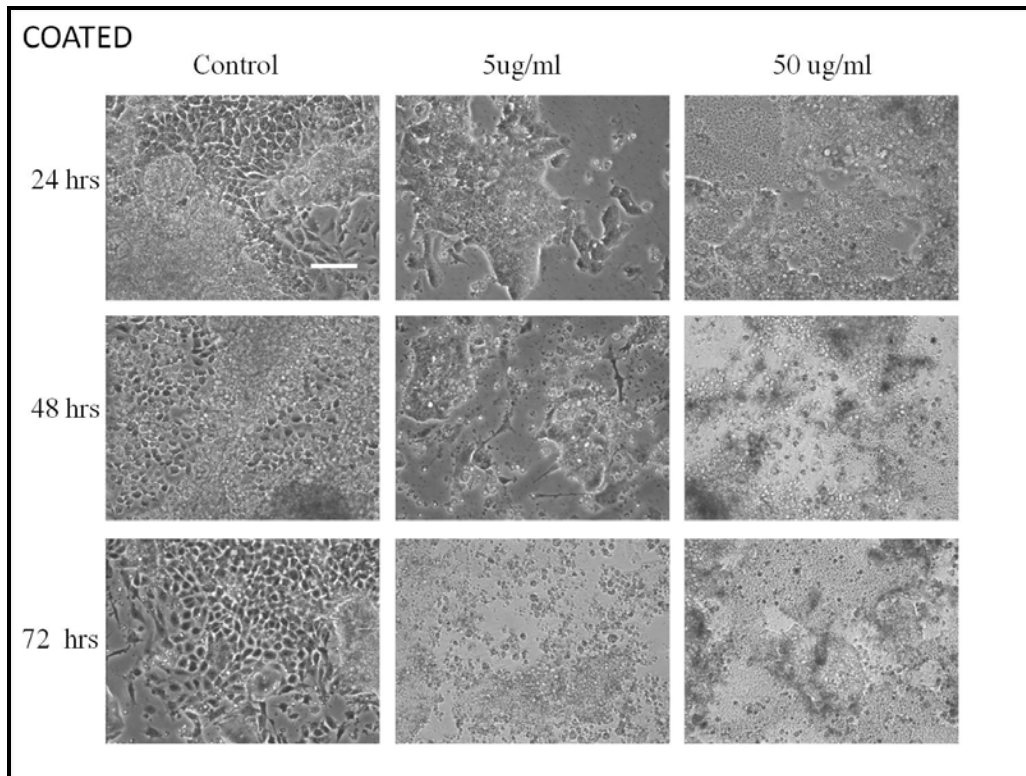


Figure 2.10: Ag Np's inhibit spontaneous MES cell differentiation even after LIF withdrawal. MES cells were treated with coated (top panel) and uncoated Ag Np's (bottom panel). Left column is control MES cells, centre column is 5 $\mu\text{g}/\text{ml}$ and right column is 50 $\mu\text{g}/\text{ml}$ treatments. First, second and third row in both panels represent 24 hrs, 48 hrs and 72 hrs time point respectively. The images were captured at 20 X magnification using an inverted microscope (Nikon TS 1000) and Metamorph software. Scale bar is 100 μm .



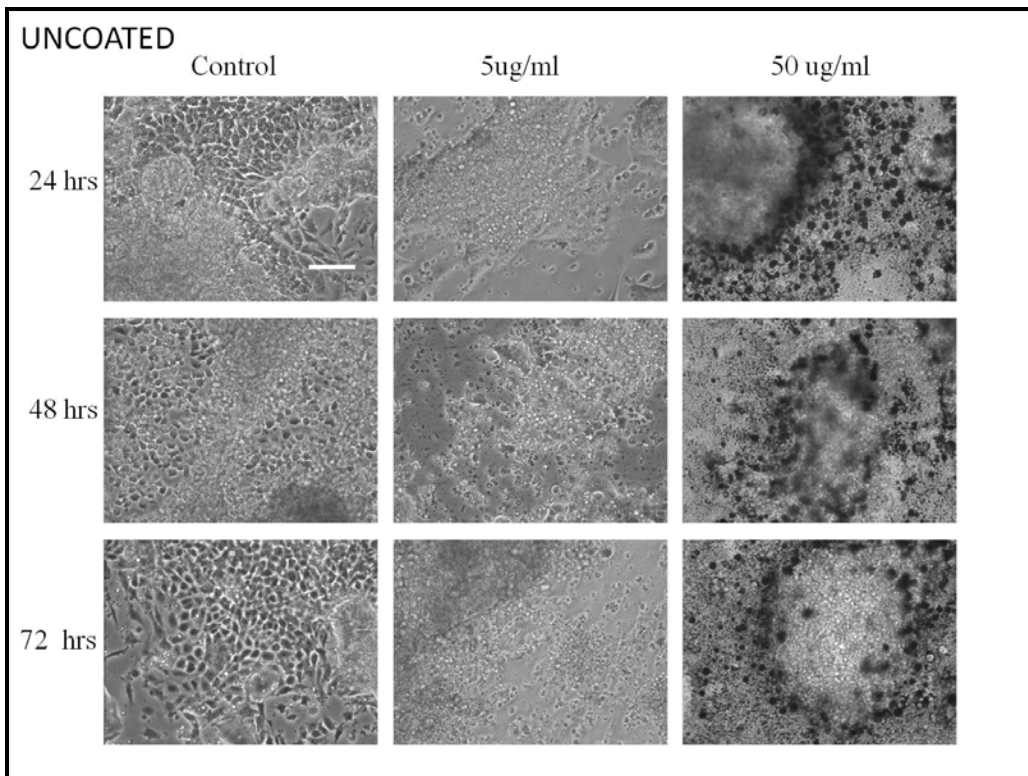
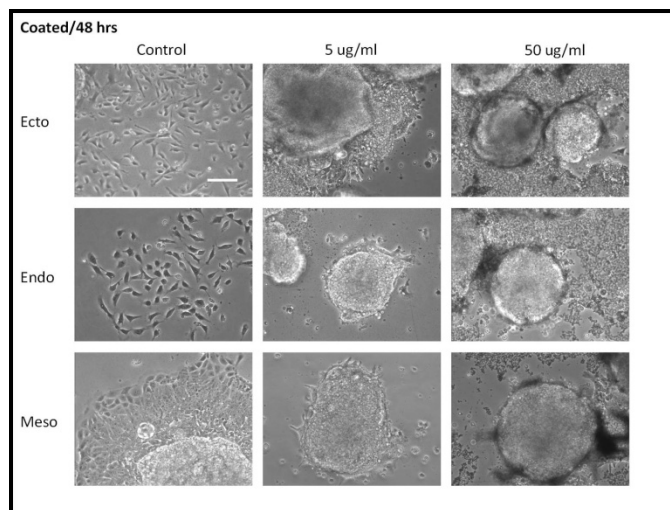
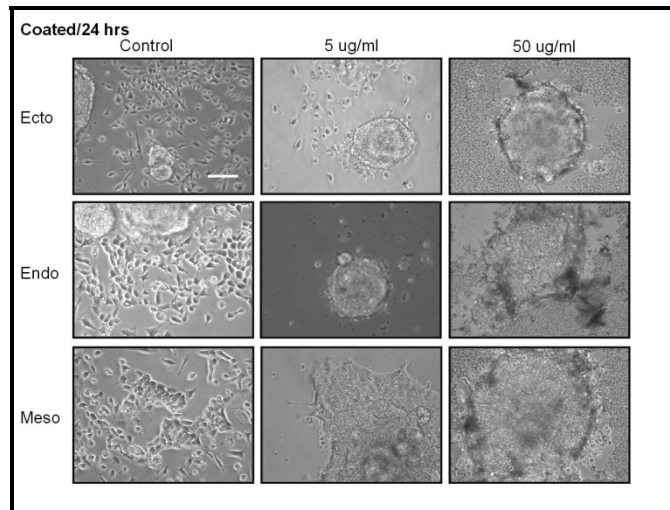


Figure 2.11a: Coated Ag Np's inhibit EB differentiation into all the 3 germ layers. Top, centre and lower panel represent 24 hrs, 48 hrs and 72 hrs time points respectively. Left column is control MES cells, centre column is 5 $\mu\text{g/ml}$ and right column is 50 $\mu\text{g/ml}$ treatments. Top row showing ectoderm differentiation, centre row showing endoderm differentiation and bottom row showing mesoderm differentiation for all panels. Images were captured at 20 X magnification using a confocal microscope (Olympus FV 1000) and Metamorph software. Scale bar is 100 μm .



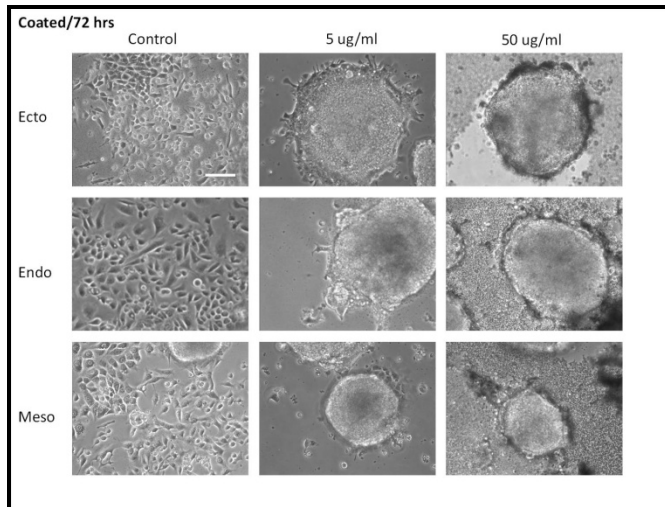
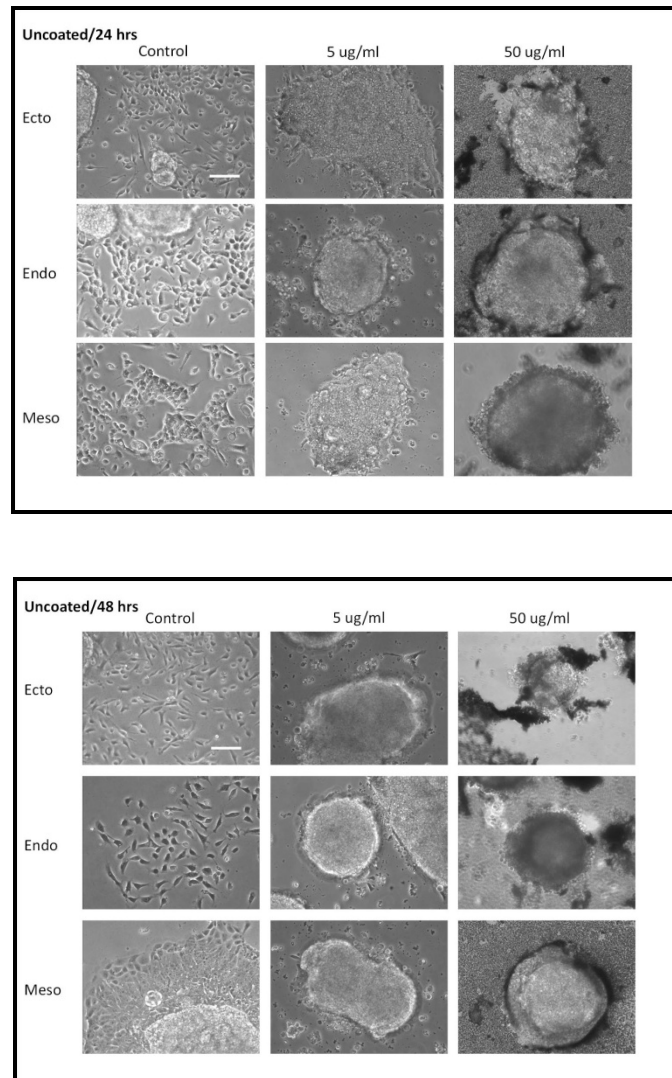


Figure 2.11b: Uncoated Ag Np's inhibit EB differentiation into all the 3 germ layers. Top, centre and lower panel represent 24 hrs, 48 hrs and 72 hrs time points respectively. Left column is control MES cells, centre column is 5 $\mu\text{g/ml}$ and right column is 50 $\mu\text{g/ml}$ treatments. Top row showing ectoderm differentiation, centre row showing endoderm differentiation and bottom row showing mesoderm differentiation for all panels. Images were captured at 20 X magnification using a confocal microscope (Olympus FV 1000) and Metamorph software. Scale bar is 100 μm .



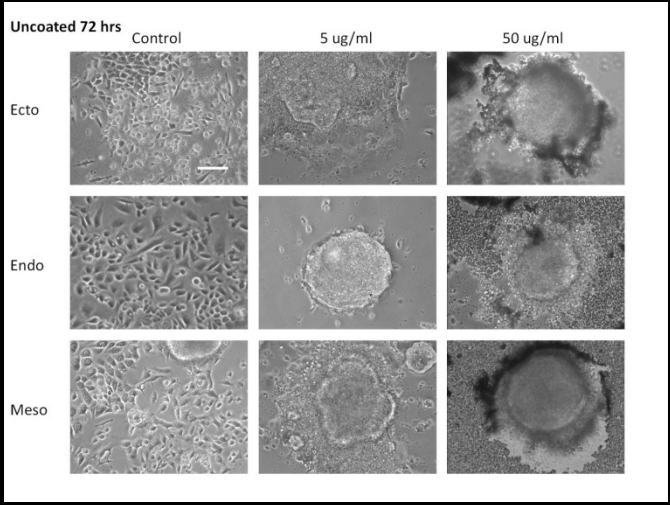
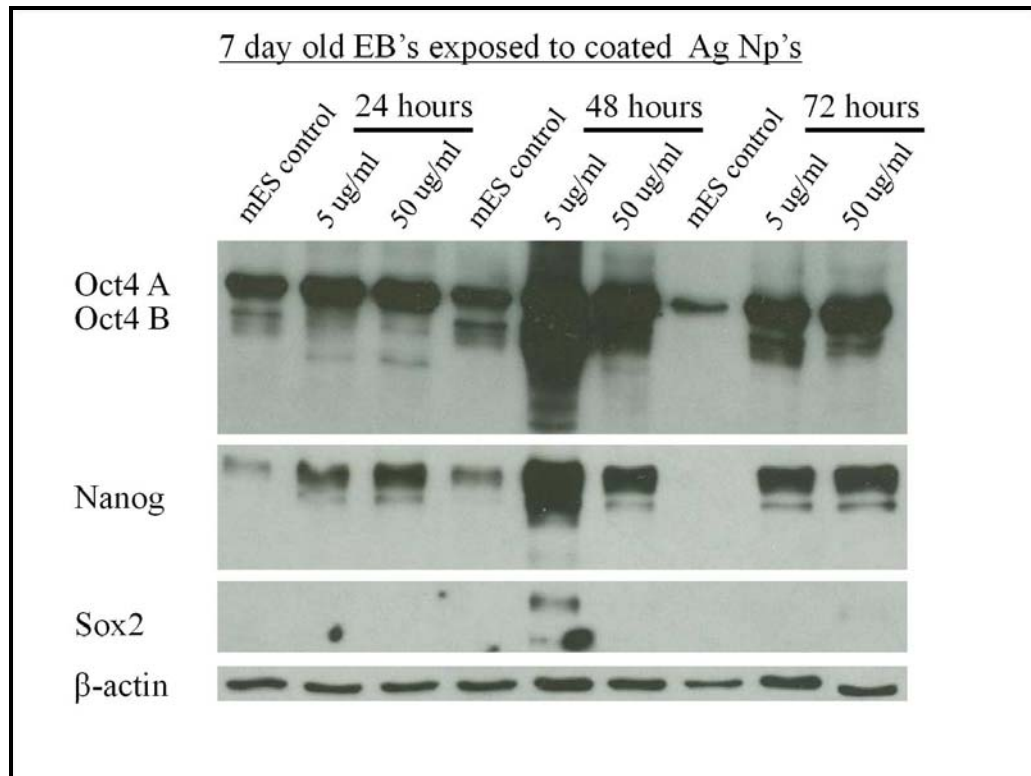


Figure 2.12: Western Blotting of pluripotency factors after Ag Np treatment. Western Blot analysis reveals coated and uncoated Ag Np's induce Oct4 and Nanog expression in 7 day old EB's exposed to 5 and 50 μ g/ml concentrations for 24 hrs, 48 hrs and 72 hrs and inhibit differentiation. Top panel represents coated Ag Np's and bottom panel represents uncoated Ag Np's. β -actin was used as a loading control.



7 day old EB's exposed to Uncoated Ag Np's

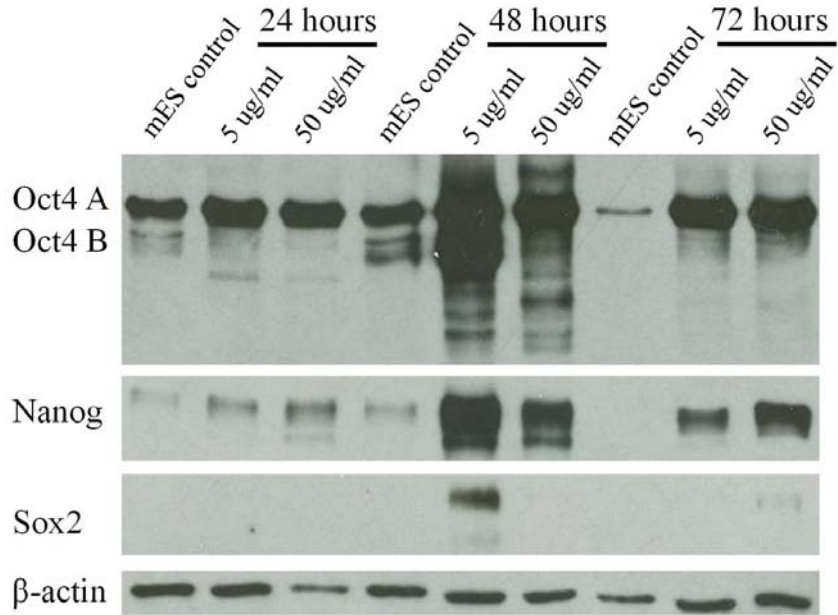
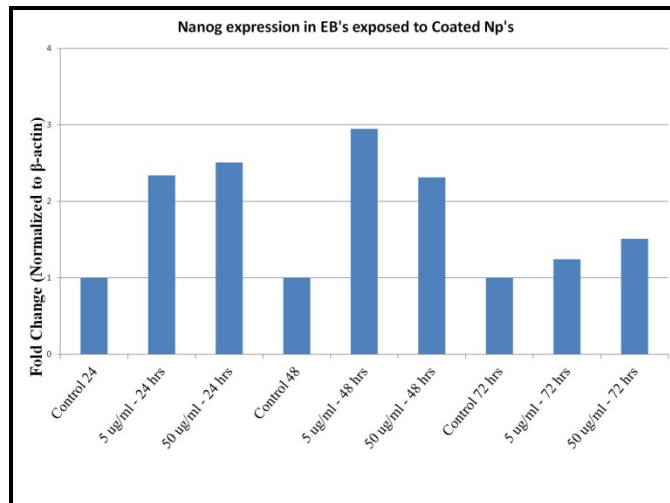
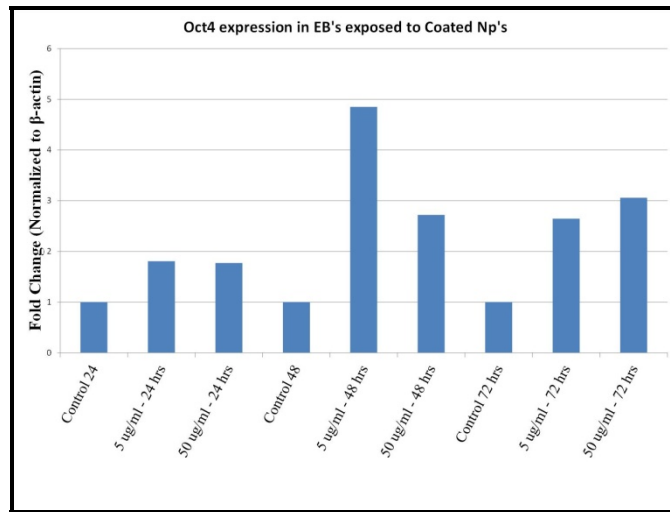


Figure 2.13a: Densitometry analysis of pluripotency factors in EB's after coated Ag Np treatment. Densitometry analysis of MES cells exposed to coated Ag Np's showing increased Oct4 and Nanog expression in 7 day old EB's exposed to 5 and 50 μ g/ml concentrations for 24 hrs, 48 hrs and 72 hrs. Protein expression was normalized to β -actin. Top panel - Oct4, centre panel - Nanog, lower panel - Sox2. Microsoft Excel was used for data analysis.



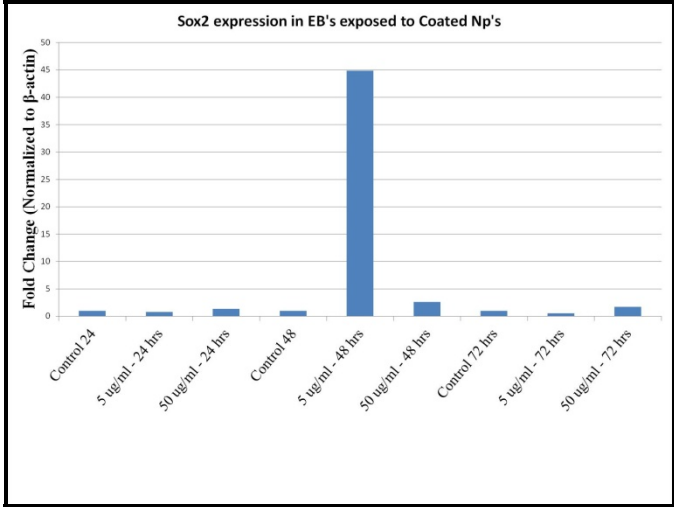
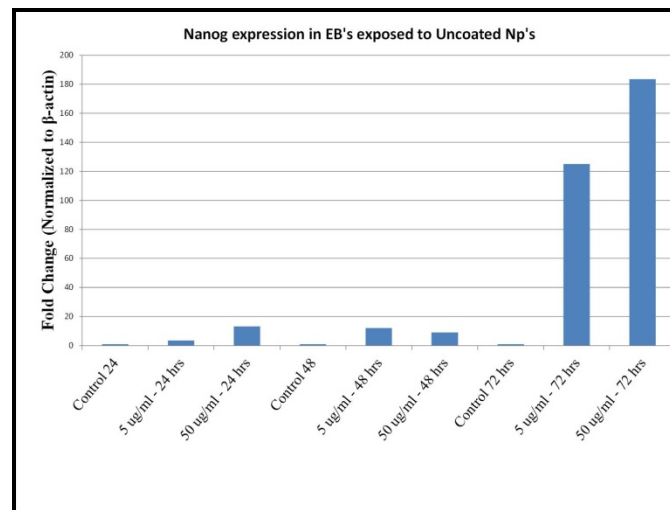
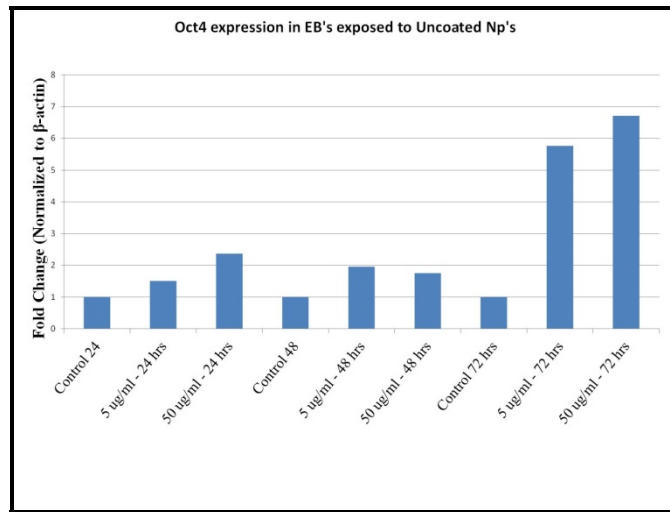
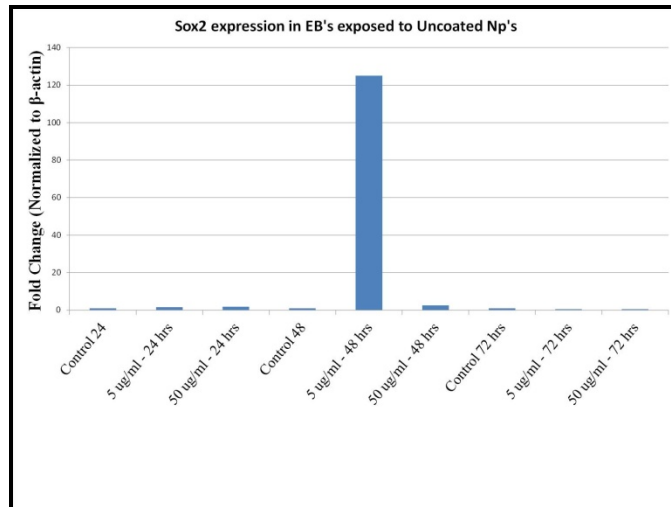


Figure 2.13b: Densitometry analysis of pluripotency factors in EB's after uncoated Ag Np treatment. Densitometry analysis of MES cells exposed to uncoated Ag Np's showing increased Oct4 and Nanog expression in 7 day old EB's exposed to 5 and 50 μ g/ml concentrations for 24 hrs, 48 hrs and 72 hrs. Protein expression was normalized to β -actin. Top panel - Oct4, centre panel - Nanog, lower panel - Sox2. Microsoft Excel was used for data analysis.





CHAPTER III

**CONVERSION OF MOUSE FIBROBLASTS TO SPHERE CELLS
WITH DIFFERENTIATION POTENTIAL INDUCED BY
ALBUMAXI-CONTAINING MEDIUM**

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David A. King¹, Yiling Hong¹

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Running Title: Reprogramming of mouse fibroblasts to other cell lineages

Key Words: Reprogramming, Transdifferentiation, Intermediate Cells, Sphere cells,
AlbuMAX I-containing medium, KSR

ABSTRACT

The reprogramming of fibroblasts to pluripotent stem cells and the direct conversion of fibroblasts to functional neurons has been successfully manipulated by ectopic expression of defined factors. We demonstrate that mouse fibroblasts can be converted into sphere

cells by detaching the fibroblast cells by protease and then using the AlbuMAX I-containing culture medium without genetic alteration. AlbuMAX I is a lipid-rich albumin. Albumin-associated lipids arachidonic acid (AA) and pluronic F-68 were responsible for this effect. The converted colonies were positive for both alkaline phosphatase and stage specific embryonic antigen-1 (SSEA-1) staining. Global gene expression analysis indicated that the sphere cells were in an intermediate state compared with MES cells and MEF cells. The sphere cells were able to differentiate into tissues representing all three embryonic germ layers following retinoic acid treatment, and differentiated into smooth muscle cells following treatment with vascular endothelial growth factor (VEGF). The study presented a potential novel approach to transdifferentiate mouse fibroblast cells into other cell lineages mediated by AlbuMAX I-containing culture medium.

INTRODUCTION

Lineage commitment was considered an irreversible process during development until it was demonstrated that adult somatic cells can be reprogrammed after fusion with a mature oocyte. Such reprogrammed cells have been used to produce cloned animals of different species (Wakayama, Perry, Zuccotti, Johnson, & Yanagimachi, 1998; Wilmut, Schnieke, McWhir, Kind, & Campbell, 1997). Recent studies also shown that the reprogramming of mouse and human fibroblasts to a pluripotent state can be achieved *in vitro* by ectopic expression of defined factors, such as Oct4, Sox2, Nanog, c-Myc, or Klf4 (or Lin28). The DNA methylation, gene expression and chromatin state of such induced pluripotent stem (iPS) cells are very similar to embryonic stem (ES) cells (Okita,

Ichisaka, & Yamanaka, 2007; Takahashi & Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007). Furthermore, a more recent study has indicated that the expression of neural-lineage-specific transcription factors *Ascl1*, *Brn2* (also called *Pou3f2*) and *Myt1l* can directly convert fibroblasts into functional neurons *in vitro* (Vierbuchen et al., 2010). Mouse fibroblasts can be reprogrammed to an intermediate state of differentiation by chemical induction (Park et al., 2011).

These studies demonstrate that fully differentiated cells can reverse their gene expression profile to that of pluripotent stem cells or other cell lineages through genetic factors or chemical induction.

We report here that using the combination of detaching the fibroblast cells by protease and expose them in an AlbuMAX I -containing culture medium can convert mouse fibroblast cells into sphere cells with differentiation potential without requiring genetic alteration. AlbuMAX I was isolated from bovine plasma through a chromatographic separation process. It uniquely retained naturally occurring lipids associated with the purified albumin, and is therefore an excellent choice to replace serum in media formulation. AlbuMAX I-containing Knock out Serum Replacer (KSR) medium has been used for the growth and maintenance of undifferentiated stem cells successfully under serum-free conditions (Skottman et al., 2006; Sperger et al., 2003). ES cells grown in KSR-containing medium differentiate less than those grown in serum-containing medium, and the medium can improve the efficiency of establishing many ES cell lines from blastocyst, as well as increase the success rate of producing chimeric mice (Cheng, Dutra, Takesono, Garrett-Beal, & Schwartzberg, 2004). Furthermore, KSR-containing

medium has been shown to increase the efficiency of iPS cell production using the viral approach (Okada, Oka, & Yoneda, 2010).

AlbuMAX I is a lipid-rich albumin. Several albumin-associated lipids have been identified in AlbuMAX I. Among them are arachidonic acid (AA) and pluronic F-68. AA is a polyunsaturated omega-6 fatty acid 20:4(ω -6), which is one of the essential fatty acids required by most mammals. This lipid has been marketed as an anabolic bodybuilding supplement. The metabolism of AA through lipoxygenase pathways leads to the generation of several different biologically active eicosanoids, which affect diverse biological processes including cell growth, cell survival, angiogenesis, and wound healing (Bazan, 2005). Several studies also have shown that AA induces a calcium influx (Erriquez et al., 2005). Basic fibroblast growth factor (bFGF), widely used to prevent stem cell differentiation, can stimulate rapid release of AA (Fafeur, Jiang, & Bohlen, 1991). Another important albumin-associated lipid is the non-ionic surfactant pluronic F-68. Pluronic F-68 facilitates cell orientation and subsequent collagen synthesis and therefore promotes early wound healing. It has been used in early postsurgical wound healing to facilitate early attachment and enhance the growth rate of human gingival fibroblasts (Hokett et al., 2000). Pluronic F-68 can also improve plant protoplast proliferation, cell proliferation, bud induction and shoot regeneration (Kumar, Laouar, Davey, Mulligan, & and Lowe, 1991). Albumin-associated lipids have been shown to play a role in regulating human ES cell self-renewal, but little is known about the role of these lipids in cellular reprogramming (Garcia-Gonzalo & Izpisua Belmonte, 2008).

MATERIALS AND METHODS

Constitution of the AlbuMAX I-containing Culture Medium

AlbuMAX I-containing stem cell medium consists of basal media and AlbuMAX I (12.5g/L, Invitrogen 11020-021). The basal media contains DMEM/F12 (Invitrogen 11330-032), 1x non-Essential Amino Acids (Invitrogen 11140-050), 1x L-Glutamine (Invitrogen 25030-018), 0.1mM β -mercaptoethanol, 4ng/ml bFGF (13256-029), thiamine (9mg/L, Sigma T1270), reduced L-glutathione (1.5mg/L, Sigma G6013), ascorbic acid -2-PO₄ (50mg/L, Sigma A8960), transferrin (8mg/L, Sigma Tobb5), insulin (10mg/L, Sigma I6634), and Recombinant Leukemia inhibitory factor (ESGRO[®], Chemicon ESG1107).

The Knockout Serum Replacer (KSR) contains DMEM/F12 (Invitrogen 11330-032), 1x non-Essential Amino Acids (Invitrogen 11140-050), 1x L-Glutamine (Invitrogen 25030-018), 0.1mM μ -mercaptoethanol, and 4ng/ml bFGF (13256-029) and 20% Knockout Serum Replacer (10828-028) (Price, Goldsborough, & Tikins,).

The Procedures of Conversion of Fibroblast Cells into Sphere Cells with AlbuMAX

I-containing Medium

Mouse embryonic fibroblast (MEF) cells were ordered from Millipore (EmbryoMax[®] primary mouse embryo fibroblasts, Neo resistant, not Mitomycin C treated, strain FVB/N, passage 3. Cat # PMEF-NL). The YFP Rosa26 MEFs were derived from a mouse carrying a YFP transgene derived from Andras Nagy's ES cell line YC5 mated to the Rosa26 mice originally made by Phil Soriano (Friedrich & Soriano, 1991;

Hadjantonakis & Nagy, 2000). To convert MEF cells into sphere cells with the AlbuMAX I-containing stem cell medium, the early passage (< 5) of MEF cells were cultured in DMEM Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Bovine Serum (FBS) at 37°C, 10% CO₂ in a 10 cm culture plate until confluent. The cells were trypsinized by adding 1.5 ml of trypsin-EDTA (Invitrogen 25200) and removing the trypsin completely within 1 minute. The trypsin-treated cells were incubated at room temperature for 2-3 minutes until they started to detach. The cells were then directly suspended in 3 ml AlbuMAX I-containing medium. The cell suspension was mixed and 0.5 ml of the cell suspension was added (about 10⁵) to a 6-well plate, each well containing 3 ml AlbuMAX I-containing medium. The cells were cultured at 37°C, 10% CO₂. The AlbuMAX I -containing medium promoted the aggregation of the small round cells into granulated cells. Some of the granulated cells continued to grow into round, bright-edged sphere cells. The converted colonies could be cultured for many passages with or without feeder.

Karyotyping

Standard G-banding chromosome analysis was carried out by the Cell Line Genetics Company (Madison, WI).

Alkaline-phosphatase (AP) and Immunofluorescent Staining

An AP detection kit (Chemicon SCR004) was used to examine the stem cell surface marker alkaline-phosphatase expression. The colonies were fixed with 4% paraformaldehyde after incubation for 1 minute. The fixed colonies were rinsed with 1x

TBST (20 mM Tris-HCl, pH 7.4, 0.15 NaCl, 0.05% Tween-20) and stained with 0.5 mL Naphthol/Fast Red Violet Solution in the dark at room temperature for 15 minutes. The images were captured by inverted microscope (Olympus CK2) at 20X magnification.

Immunofluorescence Staining for SSEA1 Markers

The coverslips with converted colonies were fixed with Formalde-Fresh (Formaldehyde 4%W/V, Methanol 1%W/V), permeabilized in PBS containing 1% NP40, and blocked with 10% horse serum for 1 hour. The coverslips with the cells were stained with SSEA 1 (Chemicon MAB4301) at 4⁰C overnight. After washing with TBST, the cells were stained with fluorescent-labeled secondary antibody Alexa Fluor 488 (1:400, from Molecular Probes). The coverslips were mounted with Gelmount (Fisher Scientifics). Images were acquired with an inverted microscope (Nikon TS100) at 20X magnification using MetaMorph Imaging Software.

Microarray Analysis

Microarray studies were processed by Asuragen, Inc. MEF, mouse ES cell J11, and two weeks old sphere cells total RNA were isolated from these cell lines, according to the company's standard operating procedures. The purity and quantity of total RNA samples were determined by absorbance readings at 260 and 280 nm using a NanoDrop ND-1000 UV spectrophotometer. The integrity of total RNA was qualified by Agilent Bioanalyzer 2100 capillary electrophoresis. Total RNA (300 ng per sample) was used for preparation of biotin-labeled targets (cRNA) using a MessageAmpTM II-based protocol (Ambion Inc., Austin, TX) and one round of amplification. The cRNA yields were quantified by UV

spectrophotometry and the distribution of transcript sizes was assessed using the Agilent Bioanalyzer 2100 capillary electrophoresis system. Labeled cRNA was used to probe MouseWG-6 v1.1 Expression BeadChips, hybridization, washing, and scanning of the Illumina arrays were carried out according to the manufacturer's instructions. Raw data were extracted using Illumina BeadStudio software v 3.0. Following quality assessment, data from the replicate beads on each array were summarized into average intensity values and variances in an Excel report containing the project description (sample key), gene identifiers and corresponding probe IDs, table of detection p -values, and table of background-subtracted data. The background subtraction, expression summary, normalization, and log base 2 transformations of gene signals were carried out using Quantile Normalization (Bolstad, Irizarry, Astrand, & Speed, 2003). For statistical analysis, one-way ANOVA was used for multiple group comparison across all samples in the experiment, followed by multiple testing corrections to determine the false discovery rate. Genes with a FDR-adjusted p -value of < 0.05 were considered differentially expressed genes (DEG).

***In vitro* Differentiation Assay**

To investigate whether the sphere cells have a differentiation potential, embryoid body (EB)-like colonies were formed from two weeks converted cells by culturing the cells in AlbuMAX I-containing medium without passing for two weeks. EB-like colonies continued to differentiate on gelatin coated plates and induced by 2 μ M trans-retinoic acid for an additional 10-15 days. Expression of endoderm-, mesoderm-, and ectoderm-specific markers was examined by using antibodies raised against α -fetoprotein (1:100,

Sigma Inc. A8452), smooth muscle actin (1:100, Sigma Inc. A5228), and β -tubulin III (1:100, Sigma, Inc. T5201), and Troponin C (1:100, Santa Cruz SC-48347), respectively, at 4 °C overnight. After washing with TBST, the cells were stained with fluorescent-labeled secondary antibody Alexa Fluor 488 (1:400, Invitrogen A31620). The coverslips were mounted with vecta shield (Vector Laboratory, H 1000). Images were acquired with an inverted microscope (Nikon TS100) at 20X magnification using MetaMorph Imaging Software.

For smooth muscle differentiation, EB-like colonies were transferred to collagen IV-coated plate that contained vascular endothelial growth medium which contained 500 ml EGM-2 (Lonza CC-4173), 10 ml FBS (Lonza CC-4101A), 0.2 ml Hydrocortisone (Lonza CC-4112), 2 ml hFGF-B (Lonza CC-4113A), 0.5 ml VEGF (Lonza CC-4114A), 0.5 ml R3-IGF-1 (Lonza CC-4115A), 0.5ml hEGF (Lonza CC-4317A), 0.5 ml GA-1000 (Lonza CC-4381A), 0.5 ml Heparin (Lonza CC-4396A), and supplemented with 50ng/ml vascular endothelial growth factor (VEGF) (494-VE/CF R&D Systems) (Schenke-Layland et al., 2008). Two weeks after culturing the converted cells in this medium at 37 °C, 10% CO₂, the cell morphologies were examined through immunofluorescent staining with smooth muscle actin antibodies (Sigma Inc. A1978).

RESULTS

AlbuMAX I -Containing Culture Medium Promoted the Conversion of the Fibroblast Cells into Sphere Sells

To investigate the possible role of lipid-rich albumin in promoting the conversion of fibroblast cells into other cell lineages, mouse embryonic fibroblast cells (MEFs) cells

were cultured in a fibroblast growth medium until confluent. The cells were detached by trypsin and exposed to the AlbuMAX I-containing medium. Within a few hours, the medium promoted the aggregation of the small round cells into bright edged granulated cells as shown in Figure. 1A. Twenty-four hours later, some of the granulated cells grew into colonies. The conversion efficiency was between 50-80% (sphere cells/total cells). The colonies can be incubated and passed for many passages, with media changed every 3-5 days. The conversion process was repeated by using primary dermal fibroblasts derived from Rosa 26 YFP mouse adult skin indicated that sphere cells were not came from the rare stem cell like cells were present in the mouse embryonic fibroblast (Figure 1B). Neomycin PCR results indicated the converted cells were indeed derived from the Neo resistant MEF cells (Figure 1C). The karyotype of the converted cells indicated that there were no major translocations, amplifications or other chromosomal changes after the conversion (Figure 1D). These results indicate that an AlbuMAX I-containing medium has the ability to convert fibroblasts into sphere cells. In addition, detaching the MEFs with proteases such as trypsin or Accutase before adding the AlbuMAX I-containing medium played a very important role in the conversion process. If the fibroblast growth medium was simply replaced with AlbuMAX I-containing medium without trypsinization, the cells retained a fibroblast morphology (Figure 2A). The conversion process was also very sensitive to cell density. Cell conversion occurred at higher rates in cell densities of 10^5 /ml to 10^6 /ml cells in each well of the six-well plate. However, a cell density exceeding 10^7 /ml inhibited the conversion process (Figure 2B). Furthermore, the conversion process was also sensitive to serum and gelatin-coated plates. After trypsinization, if the cells were exposed to a small amount of serum-

containing medium and then transferred to AlbuMAX I-containing medium, conversion was not observed (Figure 2C). In addition, the conversion appeared to favor the more acidic conditions of 10% CO₂ as opposed to 5% CO₂ (result not shown).

Albumin-Associated Lipids Arachidonic Acid (AA) and Pluronic F-68 were the Critical Components in AlbuMAX I that are Responsible for the Conversion Effect

An AlbuMAX I-containing medium consists of a combination of basal medium that contains DMEM/F12, non-Essential amino acids, L-Glutamine, β-mercaptoethanol, thiamine, reduced glutathione, ascorbic acid-2-PO₄, transferrin, insulin, trace elements, bFGF and AlbuMAX I (Price et al.,). To further identify the key components promoting the conversion of MEF cells into sphere cells, we constituted the AlbuMAX I-containing medium and eliminated individual components. Results from these experiments indicated that AlbuMAX I was the critical component to convert the fibroblasts into sphere cells (Table 1).

AlbuMAX I is lipid-rich bovine serum albumin (BSA). To identify if the BSA or the lipids associated with BSA are responsible for the effect of conversion, the same amount of the low-lipid albumin, Cohn fraction V from Roche (9048-46-B) and human recombinant serum albumin (HAS, Valley Biomedical Corp Inc., HS1021) were added to the basal medium. Low-lipid albumin from Roche or human recombinant serum albumin resulted in a very limited occurrence of conversion (Figure 3A). The results indicated that albumin-associated lipids, not the albumin apoprotein, were responsible for the conversion effect. The next task was to identify the active lipids in AlbuMAX I responsible for the conversion. The low-lipid BSA was supplemented with several

albumin-associated lipids with the concentration similar to naturally retained lipids associated with the purified albumin, such as arachidonic acid (AA) (2 mg/L), stearic acid (10mg/L), myristic acid (10mg/L), linoleic acid (10mg/L), oleic acid (10 mg/L), palmitic acid (10 mg/L), palmitoleic acid (10 mg/L), and a non-ionic surfactant pluronic F-68 (7.5g/L). The results indicated that pluronic acid F-68 has the ability to promote aggregation of the detached cells (Figure 3A). Addition of AA promoted the aggregated cells to form bright and shiny edge colonies similar to the AlbuMAX I-induced colonies. The conversion effect can be fully recapitulated with KSR stem cell culture medium from Invitrogen which contains AlbuMAX I. Supplemented KSR with AA (2 mg/L) and pluronic acid F-68 (7.5g/L) enhanced the conversion effect (Figure 3B).

Characterization of the Gene Expression Profile of Sphere Cells Induced by AlbuMAX I-Containing Medium

Up-regulation of fibroblast growth factor receptors 3 (FGFR3) plays an important role in the reprogramming of primordial germ cells into stem cells (Durcova-Hills, Adams, Barton, Surani, & McLaren, 2006). We observed the up-regulation of the expression of fibroblast growth factor receptor 3 (FGFR3) in a time-dependent manner after transferring the fibroblast cells into the AlbuMAX I -containing medium (Figure 4A). Furthermore, the early pluripotent surface specific embryonic antigen-1 (SSEA-1) was up-regulated one day after the fibroblast cells exposure AlbuMAX I -containing medium (Figure 4B). The converted colonies were positive for both alkaline phosphatase and SSEA-1 staining (Figure 4C). To further characterize the gene expression profile of the sphere cells, the Illumina Microarray global gene expression analysis of the sphere cells

compared with MES cells and MEF cells indicated that the sphere cells gene expression was relatively different from both MES cells and MEF. The results indicated that the AlbuMAX I-containing medium indeed promoted the changes of gene expression in fibroblast cells to an intermediate state. In addition, we examined the expression profiles of the 24 genes identified by Yamanaka as being related to induction of pluripotency (Takahashi & Yamanaka, 2006). Seven of 24 genes were expressed with similar regulation between MES cells and sphere cells; among them, the Nanog gene is up regulated in sphere cells compared with MEF cells, but the expression of Oct4 were not changed in sphere cells compared with MEF cells (Table 2).

The Differentiation Potential of the Sphere Cells

To investigate if the sphere cells induced by an AlbuMAX I-containing medium have developmental potential, we performed differentiation assays with sphere cells. Two week old sphere cells were cultured in AlbuMAX I-containing medium without FGF, and embryoid bodies formed. The embryoid bodies can be induced to differentiate into cell types normally derived from the three embryonic germ layers by 2 μ M trans-retinoic acid for an additional 10 days on gelatin coated plates. The expression of endoderm-, mesoderm-, and ectoderm-specific markers was confirmed by using antibodies raised against α -fetoprotein, smooth muscle actin, and β -tubulin III, respectively, indicating that these sphere cells have the capacity to differentiate into different cell types (Figure 5A). In addition, the sphere cells can also be differentiated into smooth muscle cells after transferring to collagen VI-coated plate that contained EGM-2 vascular endothelial growth medium supplemented with 50ng/ml vascular endothelial growth factor (VEGF)

(Schenke-Layland et al., 2008). Two weeks after culturing the converted cells in this medium, the cell morphology and protein expression was examined by immunofluorescent staining and western blot with smooth muscle actin antibodies (Figure. 5B &5C).

DISCUSSION

We have demonstrated for the first time that a lipid-rich albumin-containing medium has the ability to convert mouse embryonic fibroblast cells into sphere cells, and these sphere cells have the potential to differentiate into other cell lineages. Sphere cells could be the intermediate early stage of iPS cells. As the sphere cells continue to be passed in the AlbuMAX I-containing medium, a stable cell line can be established. The established stable cells expressed the same level of Oct4, Sox2 and Nang as mouse embryonic stem cells, and exhibited morphology similar to that of ES cells. The established stable cells were able to proliferate well, as shown in Figure 1S. Over the course of this project, we have noticed that only a few conversions can result in establishing stable cell lines, with the majority of converted cells remaining in an intermediate stage (sphere cells), which may be due to the cells being partially reprogrammed. The partially reprogrammed intermediate cells did not express pluripotent proteins, like Oct4. This result indicated that less stringent Nanog and some other “stemness genes” might be sufficient to reprogram differentiated cells to an intermediate state; Oct 4 could be the ultimate factor that brings the cells to the complete state, a conclusion that is consistent with Dr Tian’s funding (Park et al., 2011). Interestingly, the partially reprogrammed cells can be

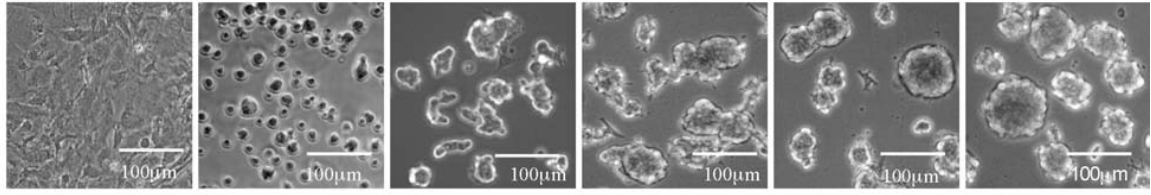
differentiated into other cell lineages *in vitro*. The avenue of promoting the fully reprogramming of the sphere cells into iPS cells remains to be explored.

The conversion efficiency of MEFs to sphere cells is between 50-80% (sphere cells/total cells). Most of the MEFs that did not convert into sphere cells underwent apoptosis. We speculate that the conversion efficiency might be due to different stages of fibroblast cell cycle. We will answer the question through the synchronization of skin fibroblast cells at the different cell cycle stages in the future. In addition, the molecular mechanism of the reprogramming is unclear at this time. Since AlbuMAX I can stimulate rapid Ca^{2+} release from the sarcoplasmic reticulum (Erriquez et al., 2005), we speculate that the Ca^{2+} influx induced by AlbuMAX I might play an important role in the conversion process. Thapsigargin, a tight-binding inhibitor for sarco/endoplasmic reticulum Ca^{2+} ATPase was added to the AlbuMAX I-containing medium at a concentration of $1\mu\text{g/ml}$ (Mircetic & Casad, 2002). As shown in Figure 2S, FGFR3 expression and the conversion of fibroblast cells into sphere cells were inhibited by Thapsigargin. These results suggested that the Ca^{2+} influx induced by AlbuMAX I might play a very important role in up regulation of the FGF signaling pathways during the conversion process. Reprogramming with lipid rich albumin could be a revolutionary method for reprogramming or transdifferentiating one cell lineage to another cell lineage in an easy and low-cost manner without genetic alteration. The methodology described has the potential to greatly accelerate cell therapy research.

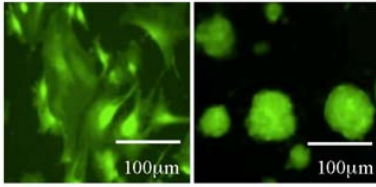
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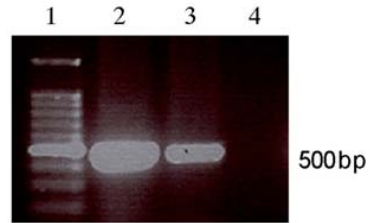
Figure 3.1: AlbuMAX I-containing medium induced the conversion of fibroblast cells into sphere cells. A. Phase contrast images document the sequential changes in morphology of MEF cells during the conversion process. From the left panel to the right: mouse embryonic fibroblast cell morphology (MEF) cultured in skin fibroblast cell medium (prior to trypsinization) or 0, 4, 8, 12, 24 hours after the trypsin-treatment and exposure to AlbuMAX I-containing medium. The images were acquired by inverted microscope (Nikon TS100) at 10X magnification using MetaMorph Imaging Software. B. Primary dermal fibroblasts derived from Rosa 26 GFP mouse adult skin was converted into sphere cells using AlbuMAX I-containing medium. C. Neomycin gene PCR analysis using the primer: Neof: ATTGAACAAGATGGATTGCAC and NeoR: TTCGTCCAGATCATCCTGATCGAC. From left panel to right, molecular weight marker, MEF cells genomic DNA, converted sphere cell line genomic DNA and mouse J11 embryonic stem cell genomic DNA. D. The G-banding chromosome analysis of MEF cells and the converted cells indicated that converted cells have a normal karyotype after conversion with AlbuMAX I-containing medium.



A MEF 0h 4h 8h 12h 24h



B



C



D MEF

Sphere cell

Figure 3.2: The culture strategy of the conversion process. A. Detachment of the MEF cells with protease is required for conversion with AlbuMAX I-containing medium. Left panel, mouse embryonic fibroblast cell morphology (MEF) cultured in the skin fibroblast cell medium; Middle panel, MEF cells converted into sphere cells 24 hours later after trypsinization and cultured in AlbuMAX I-containing medium. Right panel, the cells retained the skin fibroblast cell morphology if the skin fibroblast medium was simply replaced with AlbuMAX I-containing medium without trypsinization. B. The conversion process was sensitive to cell density. From left panel to right, the skin fibroblast cells were cultured in the skin fibroblast cell medium; and seeded at 10^4 /ml; 10^5 /ml, 10^6 /ml in AlbuMAX I-containing medium. C. The conversion process was sensitive to serum and gelatin. The left panel, the skin fibroblast cells cultured in the skin fibroblast cell medium; middle panel, MEF cells were exposed to the medium that contained serum, and then transferred to the AlbuMAX I-containing medium; the right panel, MEF cells were seeded in a gelatin coated plate.

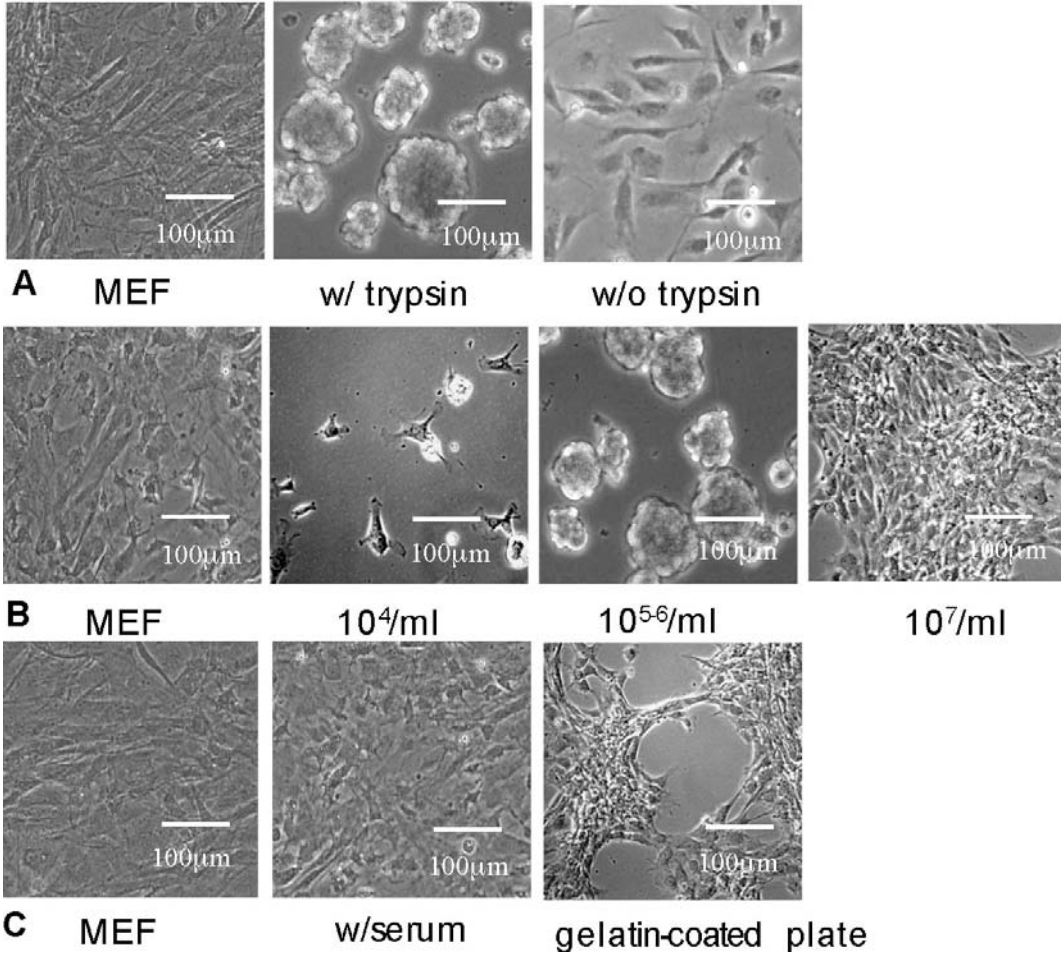


Figure 3.3: Albumin-associated lipids in AlbuMAX I was the critical components for conversion effect. A. From the left panel to the right: MEF cells were cultured in basal medium with low-lipid BSA; MEF cells were cultured in basal medium with human recombinant serum albumin; MEF cells were cultured in basal medium low-lipid BSA and supplemented with arachidonic acid (AA); MEF cells were cultured in basal medium low-lipid BSA and supplemented with pluronic F-68 (PL); MEF cells were cultured in basal medium low-lipid BSA and supplemented with arachidonic acid and pluronic F-68. B. Conversion can be recapitulated with KSR stem cell culture medium and supplemented the medium with Albumin-associated lipids enhanced the KSR conversion. From the left panel to the right: MEF cells were cultured in KSR medium; MEF cells were cultured in KSR medium supplemented with arachidonic acid (AA); MEF cells were cultured in KSR medium supplemented with pluronic F-68; MEF cells were cultured in KSR medium supplemented with arachidonic acid and pluronic F-68.

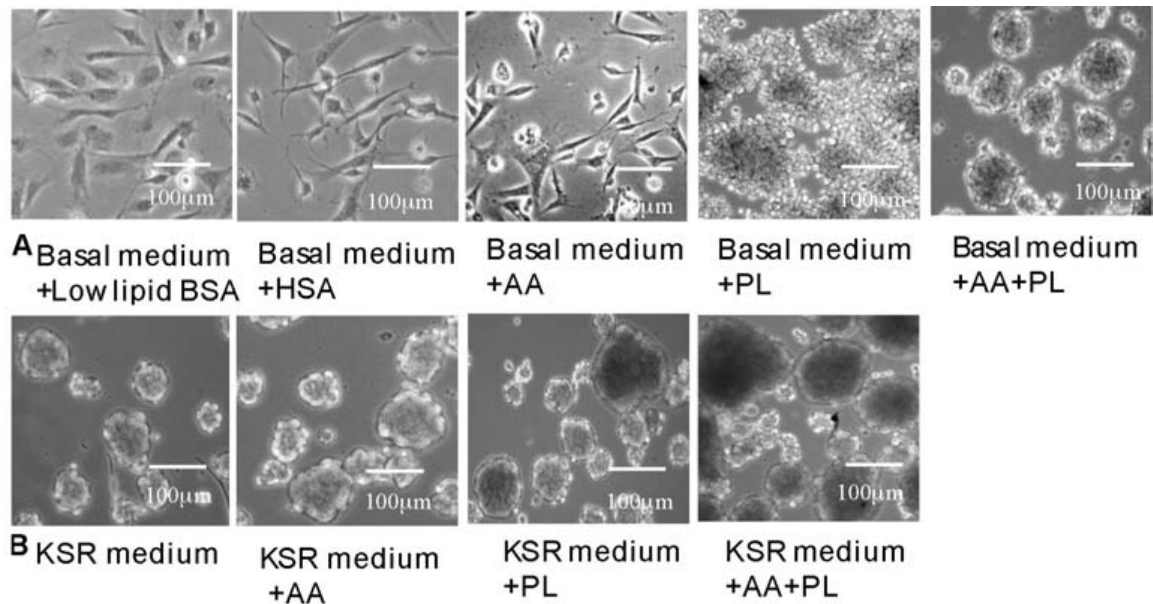


Figure 3.4: Characterization of the converted cells induced with AlbuMAX I-containing culture medium. A. FGF Receptor 3 was up-regulated induced by AlbuMAX I-containing medium. Western blotting of FGF Receptor 3 protein expression after transferring the fibroblast cells into AlbuMAX I-containing medium. Lane 1, 4 hours after culture in the medium; Lane 2, 8 hours after culture in the medium; Lane 3, 12 hours after culture in the medium; Lane 4, 24 hours after culture in the AlbuMAX I-containing medium. β -actin was used as a loading control. B. Western blotting with stem cell marker SSEA1 (Chemicon, Cat # MAB4301). Lane 1, cell lysate of fibroblast cells. Lane 2, cell lysate of 1 day old sphere cells. Lane 3, cell lysate of 2 day old sphere cells. Lane 4, cell lysate of mouse embryonic stem cells. B-actin was used as a loading control. C. Alkaline phosphatase (AP) and immunofluorescent staining for SSEA1 marker. Right panel is AP staining and left panel is SSEA1 staining. D. Heat map of gene expression array comparing 2 weeks old sphere cells to MEF cells and mouse embryonic stem cells indicated that gene expression in sphere was different from MEF cells and MES cells.

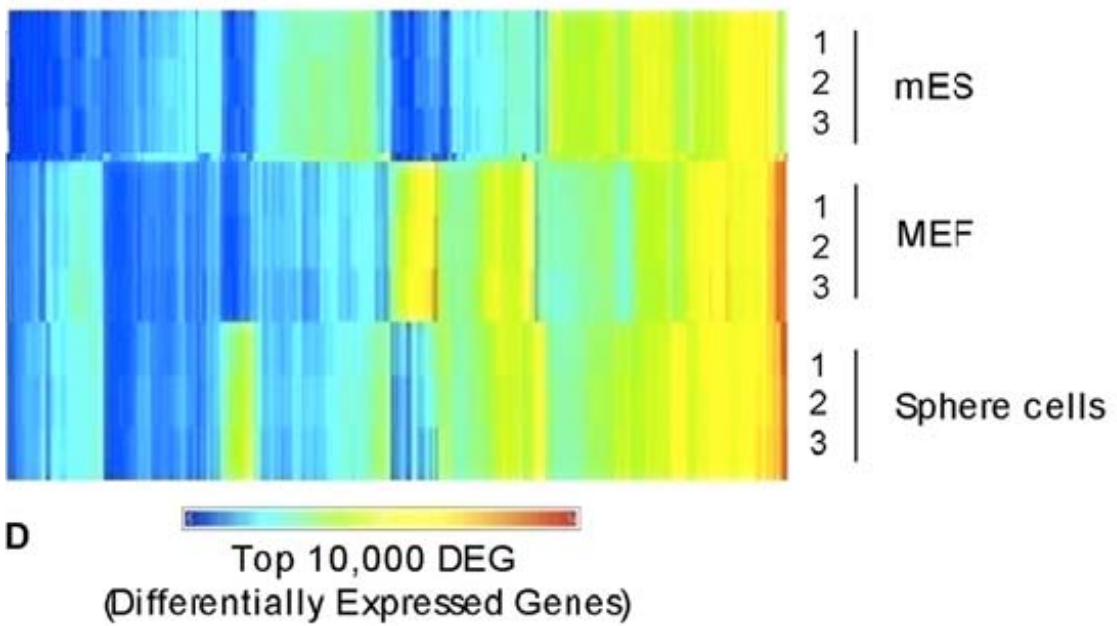
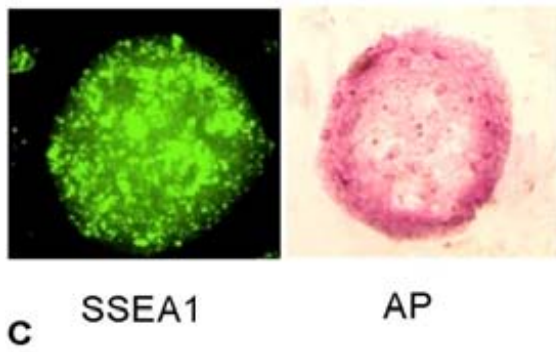
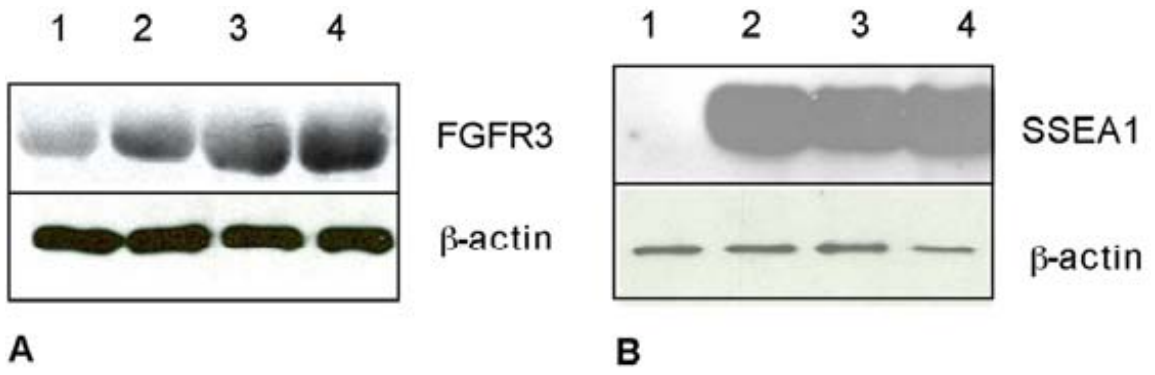
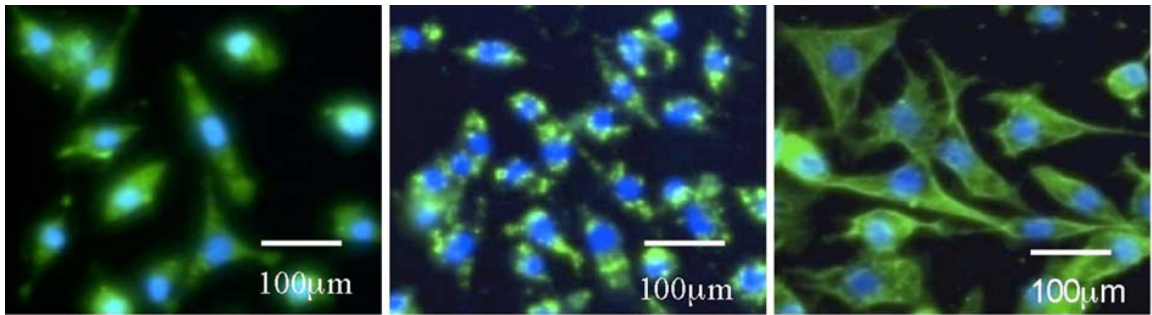


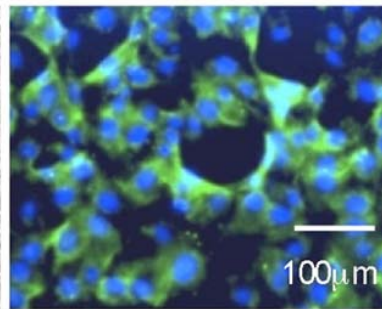
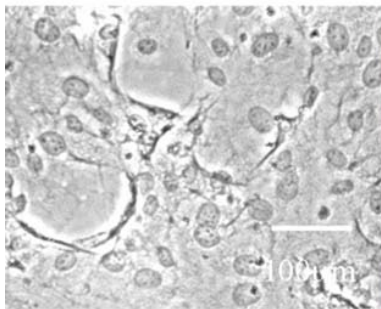
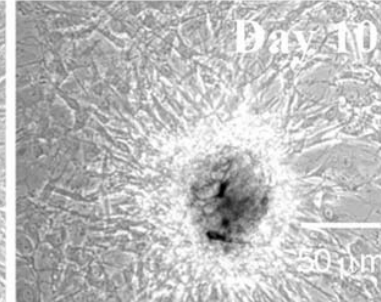
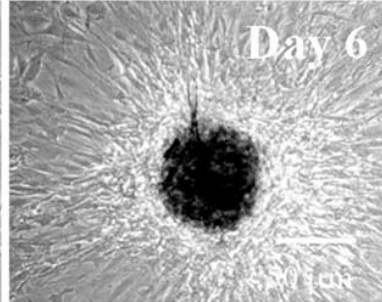
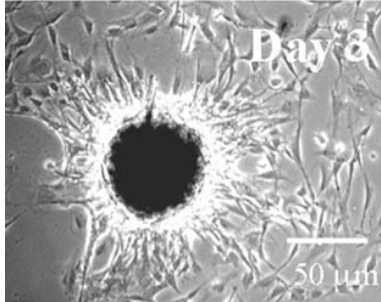
Figure 3.5: Differentiation potential of converted sphere cells. A. Embryoid bodies derived from sphere cells differentiated *in vitro* into three germ layers, induced by retinoic acid. Differentiated cells were stained with differentiation markers: endoderm α -fetoprotein (AFP), mesoderm smooth muscle (SM-Actin) (middle), and ectoderm (β -tubulin III). B. Sphere cells differentiated into smooth muscle cells induced by VEGF. Upper panel: The process of the differentiation. Lower panel: The cell morphology of smooth muscle was examined by immunofluorescent staining with smooth muscle actin antibodies. Images were acquired with an inverted microscope (Nikon TS100) at 20X magnification using MetaMorph Imaging Software. C. Smooth muscle actin expression was examined by Western blot analysis with smooth muscle actin antibodies. Lane 1, Cell lysate of MEF cells; Lane 2, Cell lysate of smooth muscle differentiated from sphere cells; Lane 3, cell lysate of mouse embryonic stem cell. β -actin used as a loading control.



A Sm-Actin

AFP

β -tubulin III



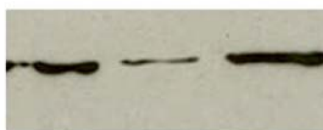
Smooth muscle cells

B

1 2 3



Smooth muscle actin



β -actin

C

Figure 3.6: The morphology of sphere cells and stable cell line cells. Phase contrast images of sphere cells and stable cell line cells were cultured in AlbuMAXI-containing medium with (right column) or without (left column) a feeder layer. Stable cells exhibited morphology similar to that of embryonic stem cells.

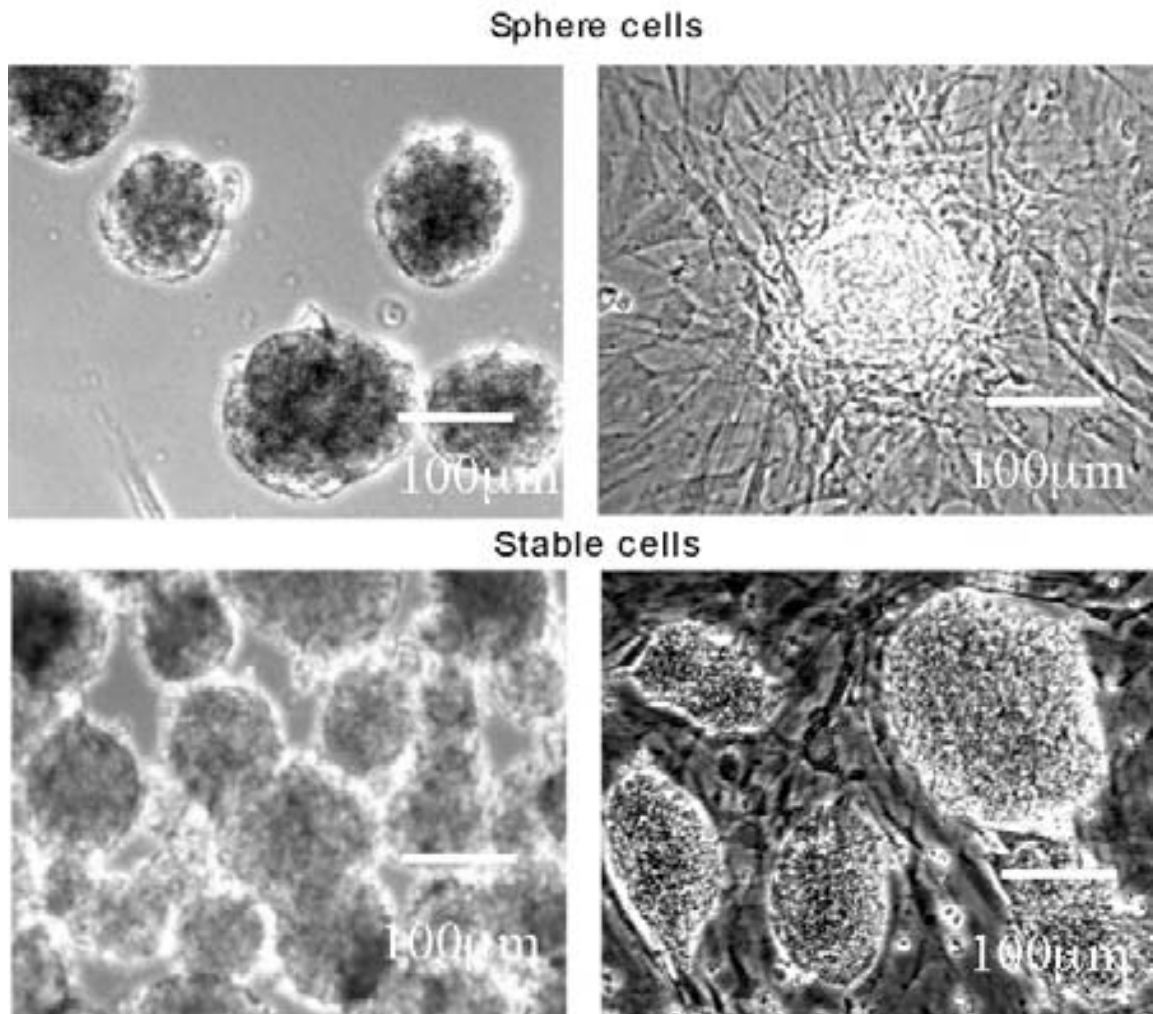


Figure 3.7: Reprogramming process and FGF Receptor 3 up-regulated induced by AlbumAX I-containing medium were inhibited by Thapsigargin, a Ca^{2+} ATPase inhibitor.

A. Thapsigargin (1ug/ml) inhibited the FGF receptor 3 expression. Lane 1, 24 hours after culture in the AlbumAX I-containing medium; Lane 2, 24 hours after culture in the AlbumAX I-containing medium with Thapsigargin (1ug/ml). β -actin was used as a loading control. B. Thapsigargin (1 $\mu\text{g/ml}$) inhibited the conversion of fibroblast cells into sphere cells. Left, AlbumAX I-containing medium; Center, AlbumAX I-containing medium with 1.5ul of DMSO; Right, AlbumAX I-containing medium with Thapsigargin (1 $\mu\text{g/ml}$), 1000X Thapsigargin stock was dissolved in DMSO. Images were acquired in the inverted microscope (Nikon TS100) at 10X magnification using the MetaMorph Imaging Software. Scale bar 100 μm .

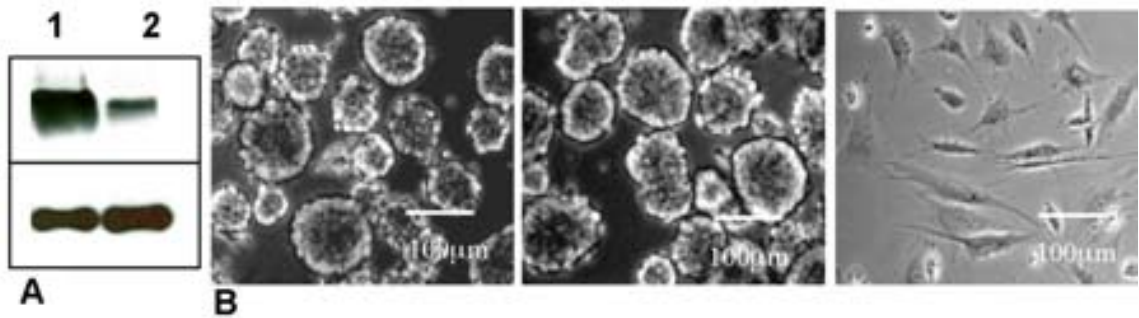


Table 3.1: AlbuMAXI is the key component in the medium promoting the conversion of skin fibroblast cells into sphere cells

Ingredient	Thiami ne	Reduced Glutathi one	Ascor bic acid-2	Transfer rin	Insul in	AlbuM AX I	bFG F	Convers ion
Concentra tion in 1X medium (mg/L)	9	1.5	50	8	10	12,500	4ng/ ml	
Medium 1	-	+	+	+	+	+	+	Yes
Medium 2	+	-	+	+	-	+	+	Yes
Medium 3	+	+	-	+	+	+	+	Yes
Medium 4	+	+	+	-	+	+	+	Yes
Medium 5	+	+	+	+	-	+	+	Yes
Medium 6	+	+	+	+	+	-	+	No
Medium 7	+	+	+	+	+	+	-	Yes

Table 3.2: The pluripotency gene expression profiles in mouse sphere cells compared with MEF cells and MES cells

Accession	Symbol	MEF cells	mESC	Sphere cells	p-value (ANOVA)*
AB093574	Nanog	11.84894582	13.10859343	13.16134754	1.7246E-06
NM_028610	Dppa4	8.05944685	9.704797769	8.321144926	6.75217E-06
NM_181548	Eras	9.183113901	10.3528649	10.28675273	0.000265692
NM_009235	Sox 15	6.959398906	7.890107949	7.587105102	0.002638022
NM_015798	Fbxo15	11.18681274	11.84775424	11.9980483	0.008136962
NM_139218	Dppa3 / Stella	5.639786313	5.932065446	5.889343914	0.06397259
NM_031261	Fthl17	5.997181891	5.992566217	5.992566217	0.233925377

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CHAPTER IV

DISCUSSION

Toxicity of silver nanoparticles in Mouse embryonic stem cells

Our results suggest that loss of self renewal capacity, decreased proliferation, altered expression of pluripotency governing factors and inhibition of differentiation are all induced by both coated and uncoated Ag Np's at 5 and 50 $\mu\text{g/ml}$ concentrations in MES cells. Therefore Ag Np's have to be assessed for toxicity in mammalian cells before commercialization and we show that MES cells can serve as an excellent model system to thoroughly characterize toxicity in the cellular and molecular levels. Recent studies have shown toxic effects of Ag Np's on mammalian and non mammalian cells by inducing cell death, DNA damage and stress response (Ahamed et al., 2010). MES cells are pluripotent cells capable of giving rise to all tissues of the body (Chambers & Smith, 2004). We show that Ag Np's induce toxicity by increasing ROS production (cell stress, due to an imbalance in antioxidant activity), Annexin V (apoptosis) expression, PI (necrosis) staining and loss of MMP in MES cells which have dynamic stress response activation signals compared to somatic cells. Prolonged exposure to Ag Np's further increased cell stress and cell death and the stress response signaling network activated to restore normal cell functions was no match for Ag Np induced toxicity. Our results correlate with other

studies showing activation of programmed cell death and increased ROS activity upon nanoparticles exposure to mammalian cells (Aarts & te Riele, 2010; Ahamed, Akhtar, Siddiqui et al., 2011; Hong, Cervantes, & Stambrook, 2006). Upon differentiation, ES cells lose AP activity and hence their pluripotency (Zhu et al., 2007). Multi walled carbon nanotubes show decreased AP activity by 24 hrs (Xing et al., 2011; Zhu et al., 2007). We show that MES cells lost AP activity after Ag Np treatment. Coated and uncoated Ag Np's induced post-translational modifications of the master regulator protein, Oct4 and Nanog, both involved in pluripotency maintenance in ES cells. Oct4 showed typical patterns of ubiquitination and Nanog showed dephosphorylation, both modifications targeting for protein degradation. Dephosphorylation of Nanog can destabilize the protein under stress conditions. We also see a slight increase in Oct4 and Nanog expression after Ag Np treatment suggesting pluripotency factors might also have a role in activating stress response signals crucial for MES cell survival. One study has shown Oct4 to be critical for survival during stress conditions and indirectly activates Survivin through signal transducers and activators of transcription 3 (STAT3) pathway (Guo, Mantel, Hromas, & Broxmeyer, 2008). Sox2 also showed increased expression along with Oct4 and Nanog (data not shown). These results suggest that the pluripotency interacting network works together during genotoxic insults in MES cells. Also, Oct4 isoforms (Oct4A and Oct4 B) are involved in governing stress related responses. Our western blots show these isoforms are also induced by Ag Np's. But further analysis using specific antibodies detecting Oct4 isoforms must be used. To our knowledge, this is the first study to show increased expression of Oct4, Nanog and Sox2 after Ag Np treatment in MES cells. Further, cells in different phases of cell cycle were altered when

compared to control MES cells due to the interaction with Ag Np's suggesting these cells lose self renewal capacity and pluripotency. Finally, differentiation potential of EB's into all 3 germ layers and spontaneous differentiation of MES cells in culture is impaired due to Ag Np's (Figure 4.1). This can be a major concern for adult stem cells exposed to Ag Np's for long time periods in the body. The regenerative capacity of stem cells will be lost and no replacement of damaged tissue due to injury or normal wear and tear of the body will be possible.

Chemical based reprogramming of somatic cells to sphere cells

We show that reprogramming with lipid rich albumin could be a revolutionary method for reprogramming or transdifferentiating one cell lineage to another cell lineage in an easy and low-cost manner without genetic alteration. The methodology described using AA and PL has the potential to greatly accelerate cell therapy research.

Reprogramming using small molecules

Generating induced pluripotent stem cells (iPS) using viral, plasmid, mRNA and protein based approaches have been successful although the efficiency of iPS cell formation is low (< 0.5%). The use of small molecules and chemicals to increase the efficiency of iPS cell generation is successful to large extent along with the above said approaches, but complete reprogramming by only using small molecules or chemicals are yet to be shown. Our study provides a basic foundation towards chemical based reprogramming. We have used small molecules which belong to several categories including DNA

methylation inhibitors, histone deacetylase inhibitors, cell cycle calcium channel regulators, and inducers of cell proliferation and are successful in partially reprogramming MEF cells to sphere cells using single or a combination of small molecules (Figure 4.2 and Table 4.1). We also induced transdifferentiation of MEF cells to neuronal cells using small molecules in specific concentrations (Figure 4.3 and 4.4), but further characterization of these converted sphere cells should be undertaken. Using viral approaches or the above said methods could cause tumorigenesis and genetic abnormalities during reprogramming to make iPS cells making it unsafe for gene therapy and tissue regeneration. Together, one can conclude that using small molecules can eliminate these concerns and revolutionize the process of generating iPS cells which could be deemed safe for treating diseases originating from the neuronal, cardiac and pancreatic lineages.

Figure 4.1: Model depicting Ag Np induced toxicity in MES cells. Ag Np's can interact with MES cells affecting stem cell renewal, pluripotency and differentiation.

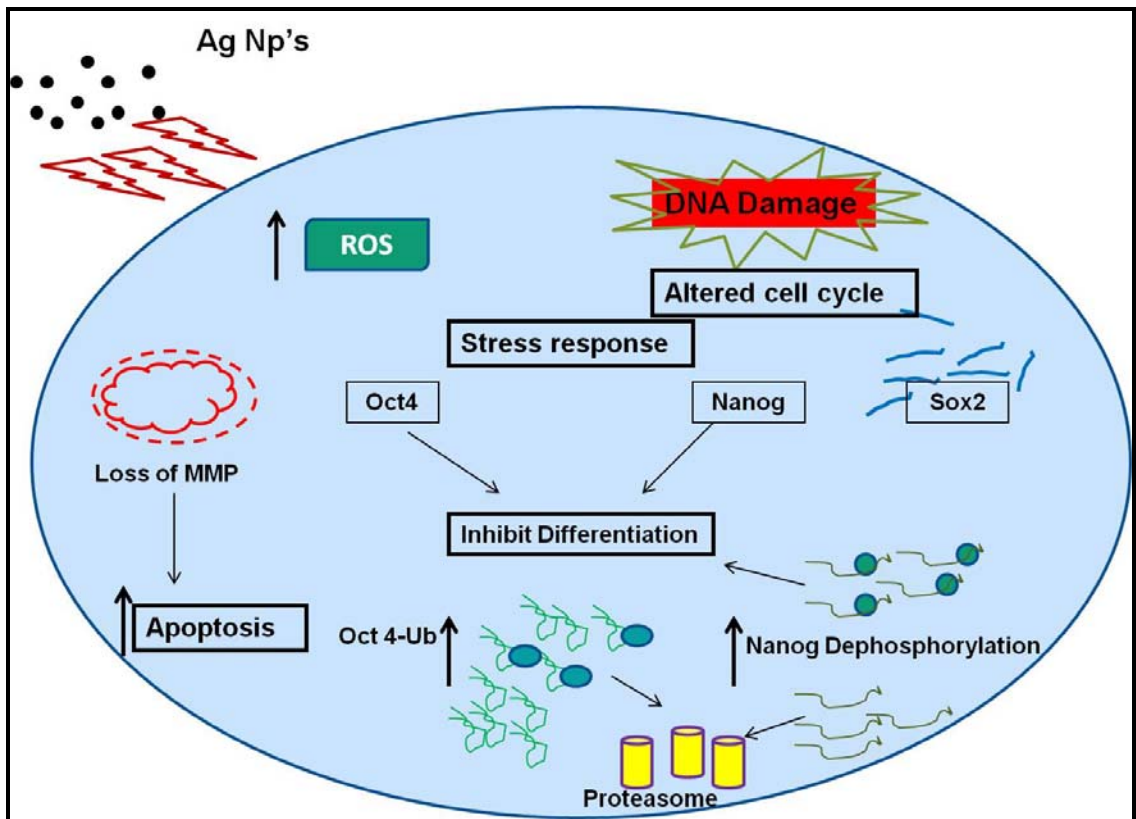


Figure 4.2: Chemical reprogramming using small molecules. Phase contrast images of initial stages of sphere cell conversion using a combination of small molecules (top panel) and single small molecules (lower panel). Images were taken at 10 X magnification and captured using an inverted microscope (Nikon TS 1000) and MetaMorph software.

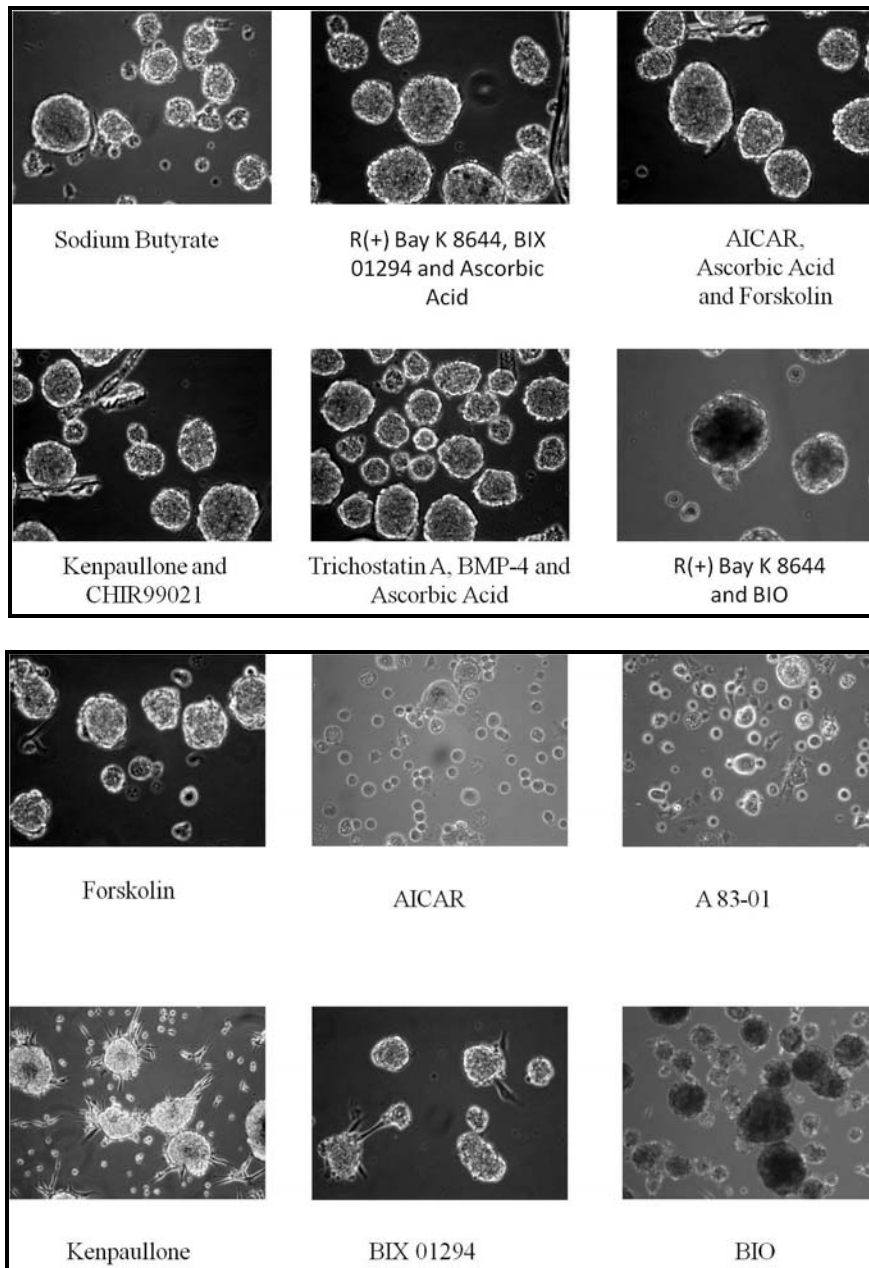


Figure 4.3: Direct reprogramming of MEF's into neuronal positive cells.

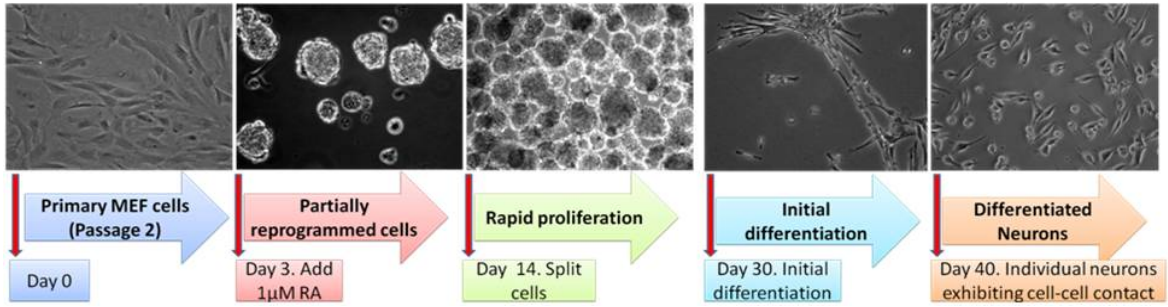


Figure 4.4: Immunostaining of neuronal cells. Directed reprogramming of MEF's to neural cells showed positive expression for TUJ1 (top left), Nestin (centre), and β -tubulin (top right). The lower panel is negative control. Draq 5 is stained blue which is specific to the nucleus. Scale bar indicates 50 μ m. Images were acquired (20X magnification) with a confocal laser scanning microscope (FV 1000).

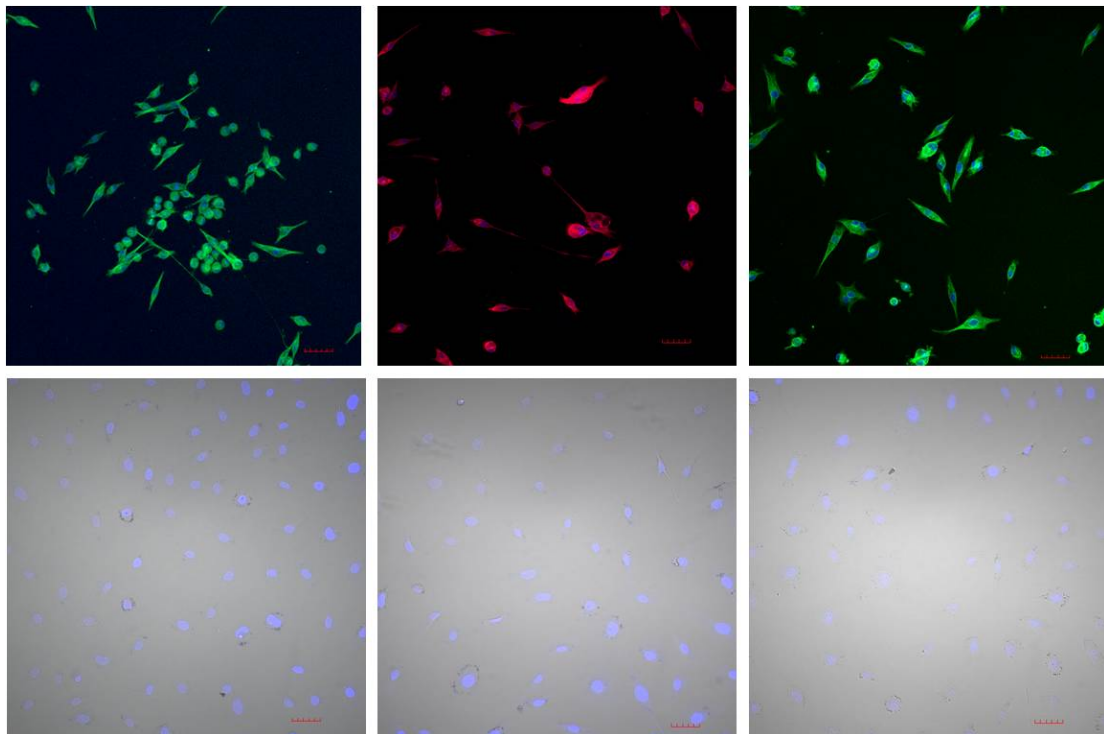


Table 4.1: List of small molecules used in the reprogramming process.

	Chemical Name	Final Concentration	Sphere cell conversion
1.	Sodium Butyrate	0.5 mM	Complete conversion
2.	SB 431542	3 μ M	Minimal/no conversion
3.	A 8301	0.5 μ M	Minimal/no conversion
4.	Forskolin	5 μ M	Complete conversion
5.	Pyrintegrin	2 μ M	No conversion
6.	RG 108	5 μ M	No conversion
7.	Y 27632	10 μ M	No conversion
8.	BIO	5 μ M	Complete conversion
9.	R (+) Bay K 8644	2 μ M	Complete conversion
10.	BIX 01294	1 μ M	Partial conversion
11.	Thiazovivin	2 μ M	Minimal/no conversion
12.	Alk 5 Inhibitor	2 μ M	Minimal/no conversion
13.	AICAR	0.5 mM	Complete conversion
14.	Trichostatin A	20 nM	Complete conversion

15.	BMP-4	10 ng/ml	Complete conversion
16.	Ascorbic Acid	20 µg/ml	Complete conversion
17.	Kenpallone	5 µM	Partial conversion
18.	CHIR 99021	10 ng/ml	Partial conversion
19.	Kenpallone CHIR 99021	5 µM 10 ng/ml	Complete conversion
20.	Trichostatin A BMP-4	20 nM 10 ng/ml	Complete conversion
21.	Trichostatin A BMP-4 Ascorbic Acid	20 nM 10 ng/ml 20 µg/ml	Complete conversion
22.	R(+) Bay K 8644 BIO	2 µM 5 µM	Complete conversion
23.	RG 108 BIX 01294	µM 1 µM	Partial Conversion

24.	R(+) Bay K 8644 BIX 01294 Ascorbic Acid	2 μ M 1 μ M	Complete conversion
25.	Forskolin Ascorbic Acid	5 μ M 20 μ g/ml	Complete conversion

CHAPTER V
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APPENDIX

ZNO NANOPARTICLES INDUCES APOPTOSIS IN HUMAN DERMAL FIBROBLASTS VIA P53 AND P38 PATHWAYS

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Abstract

The production of engineered nanoparticles is growing rapidly as the field of nanotechnology continues to expand. Zinc oxide nanoparticles (ZnO NPs) are being utilized various applications including in catalysis, electronics, biosensor, medicine, paints, sunscreens and cosmetics. Due to wide-spread application of ZnO NPs, risk assessment of this nanoparticle is of great concern. This study was designed to investigate the apoptosis induction by ZnO NPs via mitogen activated protein kinase p38 and cell cycle check point protein p53 pathways in human dermal fibroblasts. MTT-based cell viability assay showed a significant decrease in cell survivorship due to ZnO NPs exposure. Phase-contrast images demonstrated the lowering of cell density and rounding of cells exposed to ZnO NPs. Apoptosis induction by ZnO NPs was confirmed by Annexin V assay. Western blot results showed that ZnO NPs up-regulated the expression p53 and phospho-p38 proteins. Further we noted that due to Zn NPs exposure p53 protein was phosphorylated at Ser³³ and Ser⁴⁶ which is known to be phosphorylated by p38. Our results suggest that ZnO NPs have potential to induce apoptosis in human dermal fibroblasts via p53-p38 pathways.

Keywords: Zinc oxide nanoparticles; Apoptosis; p53; p38; human dermal fibroblasts

Introduction

Zinc oxide nanoparticles (ZnO NPs) are used in various products including cosmetics and sunscreens due to UV-filtering properties. Recent interest of ZnO NPs has been directed towards biosensing, cell imaging, photodynamic therapy and nanomedicine (Ahamed et al., 2011; Brayner et al., 2006; Hanley et al., 2008; Premanathan, Karthikeyan, Jeyasubramanian, & Manivannan, 2011). Despite the widespread application of ZnO NPs, there is a lack of information concerning the toxicity of these nanoparticles at cellular and molecular level.

There are studies reporting that ZnO NPs produce cytotoxic effects to various mammalian cell lines. After a 72 hour exposure to a 15 µg/ml concentration of 19 nm particles, nearly complete cell death was observed in human mesothelioma and rodent fibroblasts (Brunner et al., 2006). In neuroblastoma culture, 50 nm ZnO particle exposure at 100 µg/ml resulted in nearly 50% cell death (Jeng & Swanson, 2006). ZnO particles between 20-70 nm at 50 µg/ml decreased vascular endothelial cell survivorship by 50% (Gojova et al., 2007). Human neural cells exhibited approximately a 50% decrease in survival after a 48 hour exposure to 11 µg/ml ZnO NPs, while human alveolar epithelial cell viability was reduced in half at 24 h by 4 µg/ml of 50 nm ZnO (Lai et al., 2008; Lanone et al., 2009). A reduction in neural stem cell survivorship was observed upon 24 hour incubation with 12 µg/ml ZnO NPs (Deng et al., 2009). However, as mentioned above, cytotoxic effects were observed in human T cells only beginning at 5 mM (over 400 µg/ml) (Reddy et al., 2007). In addition, cancer T cells were demonstrated to have

around 30 times more sensitivity than normal T cells to ZnO NP toxicity (Hanley et al., 2008).

We choose human dermal fibroblast cells as a model to assess whether ZnO NPs induce toxicity because dermal fibroblasts are the most abundant type of cells in skin tissue and represent the first level of exposure to many toxicants including ZnO NPs that are currently used in sunscreens and cosmetics. The present study was designed to investigate the toxicity of ZnO NPs through three approaches. First, cytotoxic response of ZnO NPs was examined by cell viability assay and phase-contrast microscopy. Second, apoptotic response of ZnO NPs was determined by Annexin V assay. Third, immunoblot analysis was used to examine the expressions of cell cycle check point protein p53 and p38 mitogen activated protein kinase (MAPK) after exposure to ZnO NPs. The p53-p38 pathway is a key signal transduction system that mediates several extracellular signals through a cascade of protein phosphorylation. It was shown that p38 MAPK phosphorylated p53 at Ser33 and Ser46 and plays a prominent role in an integrated regulation of N-terminal that regulates p53-mediated apoptosis after UV radiation (Bulavin et al., 1999). Therefore, we further explored the interaction between p38 and p53 by examining levels of p53 phosphorylated at Ser 33 and Ser 46 in response to ZnO NPs. This study provides molecular evidence for the ZnO NP-induced apoptosis in human dermal fibroblast cells.

Materials and methods

Zinc oxide nanoparticles

ZnO NPs dry powder was purchased from Nanostructure & Amorphous Materials Inc., Houston, TX (www.nanoamor.com). The detailed characteristics provided by the supplier are as: 20 nm average particle size, 99.5% purity, 50 m²/g specific surface area, milky white color, nearly spherical morphology, 0.3-0.45 g/cm³ bulk density and 5.606 g/cm³ true density.

Characterization of zinc oxide nanoparticles

We also characterized the shape and size of ZnO NPs using transmission electron microscopy (TEM) on a Hitachi H-7600 tungsten-tip instrument at an accelerating voltage of 100 kV. Dry powder of ZnO NPs was suspended in deionized water at a concentration of 1 mg/ml, and then sonicated using a Branson-1510 sonicator bath at room temperature for 15 min at 35-40W to aid in mixing and forming a homogeneous dispersion. For size measurement, sonicated 1 mg/ml ZnO NPs stock solution was then diluted to a 50 µg/ml working solution. ZnO NPs were examined after deposition of nanoparticles suspensions onto carbon film-coated Cu grids. The advance microscopy techniques (AMT) software for the digital TEM camera was calibrated for size measurements of the nanoparticles. Information on mean size and SD was calculated using point to point method as described elsewhere (Murdock, Braydich-Stolle, Schrand, Schlager, & Hussain, 2008).

Cell culture and exposure to Zinc oxide nanoparticles

Human dermal fibroblast was ordered from Promo-cell (Cat# C-12302). Human dermal fibroblast cells were cultured at 37°C and 5% CO₂ in high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco# 11965) containing 10% fetal bovine serum, 0.5% penicillin streptomycin (Gibco# 15140), 1% glutamine (Gibco# 35050) and 1% non-essential amino acids (Gibco# 11140). Cells were passaged using 0.25% trypsin (Gibco# 25200). At 85% confluence, cells were harvested using 0.25% trypsin and were sub-cultured into 6-well plates or 96-well plates according to the selection of experiments. Cells were allowed to attach the surface for 24 h prior to treatment. ZnO NPs were suspended in cell culture medium and diluted to appropriate concentrations. The dilutions of ZnO NPs were then sonicated using a sonicator bath at room temperature for 10 min at 40W to avoid nanoparticles agglomeration prior to administration to the cells.

Cell viability

Cell viability was determined by MTT assay (CGD-1 kit, Sigma-Aldrich). Human dermal fibroblast cells (1×10^4 cells/well) were seeded into a 96-well plate (PerkinElmer SpectraPlate). After 24 h, the media were replaced with 100 µl of media prepared with ZnO NPs (2.5, 10, 25 50 and 100 µg/ml) and incubated for 24 h. After the exposure completed, the medium was removed from each well and replaced with new medium containing MTT solution in an amount equal to 10% of culture volume, and incubated for 3 h at 37 °C until a purple colored formazan product developed. The resulting formazan product was dissolved in acidified isopropanol and the absorbance was measured at at

570 nm and 690 nm by using a microplate reader (Synergy-ht, Biotek). Background absorbance at 690 nm was subtracted from absorbance at 570 nm.

Cell morphological examination

Cell morphology of human dermal fibroblasts was examined following exposure to different concentrations ZnO NPs for 4 and 24 h using a phase-contrast inverted microscope (Nikon TS100).

Apoptosis detection by Annexin V staining

We examined the apoptosis induction in human dermal fibroblasts exposed to 50 µg/ml ZnO NPs for 24 h using Annexin V staining assay. After exposure completed, cells were fixed with Formalde-Fresh (formaldehyde 4%W/V, Methanol 1%W/V), permeabilized in PBS containing 1% NP40, and blocked with 10% horse serum for 1 h. The coverslips with the cells were stained with Annexin V staining (Abcam #14196) and fluorescent-labeled secondary antibody Alexa Fluor 594 (1:400, Invitrogen# A31632). Images were acquired with a Fluoview laser scanning confocal microscope (10X magnification).

Immunoblot

For Western blot analysis cells were cultured in 6-well plates and exposed to different concentrations of ZnO NPs for 4 and 24 h. At the end of exposure, cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NPs-40, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors. Cell lysates were separated on a 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore# IPVH00010). After blocking membrane for 1 hour in TBST (20 mM Tris-HCl, pH 7.6,

136 mM NaCl, and 0.1% Tween-20) containing 3% nonfat milk, membranes were incubated at 4°C overnight with primary antibody diluted in 3% milk-TBST solution. Following incubation, the membranes were washed with TBST and incubated at room temperature for an hour with horseradish peroxidase conjugated secondary antibody. Blots were developed using Amersham ECL detection reagents (RPN2106) and Kodak BioMax Light Film (Cat # 868 9358). The following primary and secondary antibodies were used: anti-p53 (1:2000, Cell Signaling #2524), anti-p53 (1:400, Calbiochem OP03), anti-Phospho-p38 MAPK (1:1000, Cell Signaling #9215), anti-Phospho-p53 (Ser³³) (1:1000, Cell Signaling #2526), anti-Phospho-p53 (Ser⁴⁶) (1:1000, Cell Signaling #2521), anti-β-Actin (1:4000, Sigma Aldrich A1978), goat anti-mouse IgG-HRP (1:2000, Santa Cruz sc-2005) and goat anti-rabbit IgG-HRP (1:5000, Santa Cruz sc-2004).

Statistical analysis

Statistical analysis was carried out by Student's t-test. Significance was ascribed at $p < 0.05$.

Results

1.1. Zinc oxide nanoparticles characterization

Fig. 1A shows TEM image of Zn NPs. Information on mean size and SD was calculated from measuring over 100 nanoparticles in random fields of view. The mean \pm SD of Zn

NPs was 23.47 ± 5.54 nm. Fig. 1B represents the frequency of size distribution of ZnO NPs.

1.2. Effect of zinc oxide nanoparticles on cell viability

Human dermal fibroblast cells were exposed to ZnO NPs at the concentrations of 0, 2.5, 10, 25, 50 and 100 $\mu\text{g/ml}$ for 24 h and cell viability was determined using MTT assay (Fig. 2). Results have shown that ZnO NPs at a concentration of 2.5 $\mu\text{g/ml}$ did not produce significant reduction in cell viability. Cells exposed to 10 $\mu\text{g/ml}$ of ZnO NPs showed 20% reduction in cell survivorship ($p < 0.05$) while greater than 85% reduction in cell viability was observed at higher concentrations (25-100 $\mu\text{g/ml}$, $p < 0.001$) (Fig. 2).

1.3. Effect of zinc oxide nanoparticles on cell morphology

The morphology of human dermal fibroblast cells was examined after ZnO NP exposure using phase contrast microscopy. No visual differences compared to the unexposed control were observed in cell exposed for 4 and 24 h to 2.5, 5 and 10 $\mu\text{g/ml}$ of ZnO NPs (data not shown). A significant lowering of cell density and rounding of cells became visually at 50 and 100 $\mu\text{g/ml}$ of ZnO NPs after 4 h exposure (Fig. 3B & C) and even at greater extent after 24 h exposure (Fig. 3E & F).

1.4. Apoptosis induction by zinc oxide nanoparticles

In phase-contrast study we noted that due to ZnO NPs exposure cells lost their normal morphology and were detached from the surface due to cell death. To confirm that cell death was due to apoptosis we further did the Annexin V assay in human dermal

fibroblasts treated with 50 µg/ml NPs of ZnO NPs for 24 h. Results showed that ZnO NPs indeed induced the apoptosis in human dermal fibroblast cells (Fig. 4).

3.3 Zinc oxide nanoparticle induced p53 up-regulation and phosphorylation of p53 at Ser³³ and Ser⁴⁶

To understand the molecule mechanisms of apoptosis induced by ZnO NPs, differential expression of tumor suppressor protein p53 in response to varying ZnO NP concentrations was analyzed in human dermal fibroblast cells by immunoblot analysis. A significantly increased expression of p53 at 4 h after exposure to 10, 50, and 100 µg/ml of ZnO NPs (Fig. 5A, lane 1-4). Almost same level of p53 expression was observed after 24 h exposure suggesting that expression of p53 protein was highest within 4 h (Fig. 5B, lane 5-8).

Activation of p53 is modulated by protein phosphorylation, the levels of phospho-p53 (Ser³³) and phospho-p53 (Ser⁴⁶) were analyzed in HF cells. Significantly increased phosphorylation of p53 at Ser³³ was observed within 4 h exposure up to the 24 h exposure in the concentration range of 10-50 µg/ml (Fig. 6A & B). A similar pattern of expression was observed in Ser⁴⁶ phosphorylation of p53 protein in the concentration range of 10-50 µg/ml of ZnO NPs for 4 and 24 h (Fig. 7A & B).

3.4 Zinc oxide nanoparticles induced p38 MAP kinase up-regulation

The p38 is a stress-activated MAP kinase that becomes activated upon phosphorylation and is involved in apoptosis initiation (Ichijo, 1999). To further examine the effect of ZnO NPs on the expression of phospho-p38 we utilized immunoblot analysis. ZnO NP

exposure for 4 h resulted in an increase in phospho-p38 levels at 50 µg/ml and 100 µg/ml concentrations in human dermal fibroblast cells (Figure 8A). Following 24 h exposure, the phospho-p38 expression was still greater than the untreated control cells (Figure 8B). These results indicated that ZnO NP exposure induced p38 MAP kinase activation and phosphorylation of p53 at Ser³³ and Ser⁴⁶, which might play an important role in regulating p53-mediated apoptosis.

Discussion

In spite of many advantages nanotechnology, studies indicate that nanoparticles may cause hazardous effects because of their unique physicochemical properties. Although the beneficial effects of ZnO NPs have attracted considerable attention, in terms of nanomedicine, (Ahamed et al., 2011; Brayner et al., 2006; Hanley et al., 2008; Premanathan et al., 2011) the investigators have not thoroughly investigated possible hazards to the environment and human. We found that ZnO NPs exposure significantly reduced the cell viability of human dermal fibroblasts and reduction in cell viability started at approximately 10 µg/ml. Cell viability data was further supported by morphology results observed by phase contrast microscopy. The lowering of cell density and rounding observed suggest that 50 µg/ml and 100 µg/ml nanoparticle concentrations induced substantial cell death. Our cytotoxicity results were similar with those of other studies (Brunner et al., 2006; Deng et al., 2009; Gojova et al., 2007; Jeng & Swanson, 2006; Lai et al., 2008; Lanone et al., 2009; Sherr, 2004; Zhao, Xu, Zhang, Ren, & Yang, 2009).

ZnO NPs induced the up-regulation of two important molecular markers, p53 and phospho-p38. The p53 is often regarded as “the guardian of the genome” due to its integral role in preserving the integrity of the genome through prevention of mutations (Farnebo, Bykov, & Wiman, 2010). Upon the cell encountering insults, p53 arrests the cell cycle, allowing DNA repair enzymes to act, and if the damage is beyond repair, initiates apoptosis to prevent the mutation from being passed on through subsequent cell divisions. Along with the increased p53 levels, the up-regulation of phosphor-p38 indicates that ZnO NP exposure resulted in cellular stress. Furthermore, there is substantial interaction between p53 and p38, as demonstrated by a study reporting that, during cellular stress induced by UV-irradiation, p38 phosphorylates p53 at two sites, stabilizing p53 and enhancing the apoptosis response (Bulavin et al., 1999). Our immunoblot analysis indicated that phosphorylation at both of these sites (Ser³³ and Ser⁴⁶) increased within 4 h exposure to ZnO NPs. The up-regulated expression patterns of p53 and p38 proteins in human dermal fibroblasts by ZnO NPs suggest that these proteins could be excellent molecule markers to assess the geno-nanotoxicology.

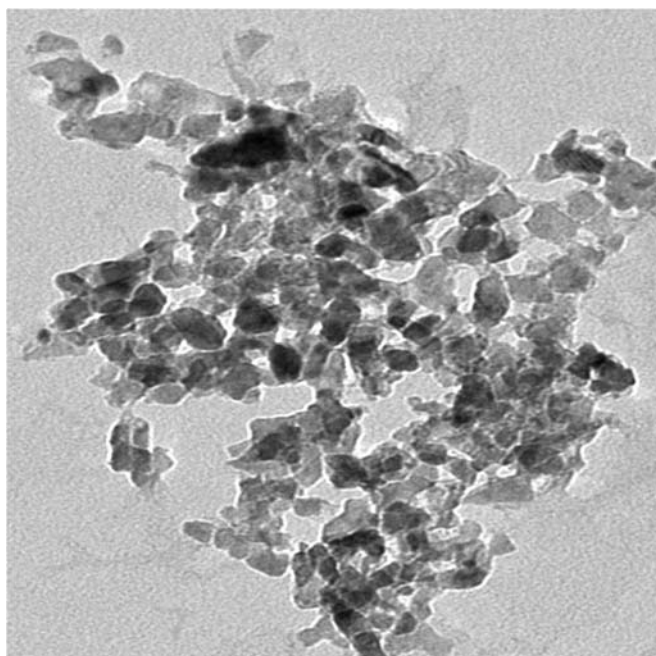
It has been well-documented that extensive DNA damage triggers apoptosis (Sherr, 2004). We examined ZnO NPs induced apoptosis in human dermal fibroblasts using annexin V assay. The results indicated that ZnO NPs significantly induced cell death/apoptosis in human dermal fibroblast cells. MTT data also revealed reduction in number of viable cells due to ZnO NPs exposure suggesting the annexin V results. We know that MTT assay measures the energy generating potential of the cell, assesses mitochondrial function by measuring electron transfer potential from there mitochondria mediated apoptosis initiated (Ahamed et al., 2008).

In summary, our data demonstrated that ZnO NPs possess apoptosis induction potential in human dermal fibroblasts which is mediated through p53-p38 pathways. These results suggest that wide-spread application of ZnO NPs should be carefully assessed as to their potential adverse effects to the environment and human.

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Figure 1. Transmission electron microscopy characterization of ZnO NPs. Figure 1A indicates that the majority of the ZnO NPs were in spherical shape. Information on mean size and SD was calculated from measuring over 100 nanoparticles. The mean \pm SD of ZnO NPs was 23.47 \pm 5.54 nm. Figure 1B represents the frequency of size distribution of ZnO NPs.



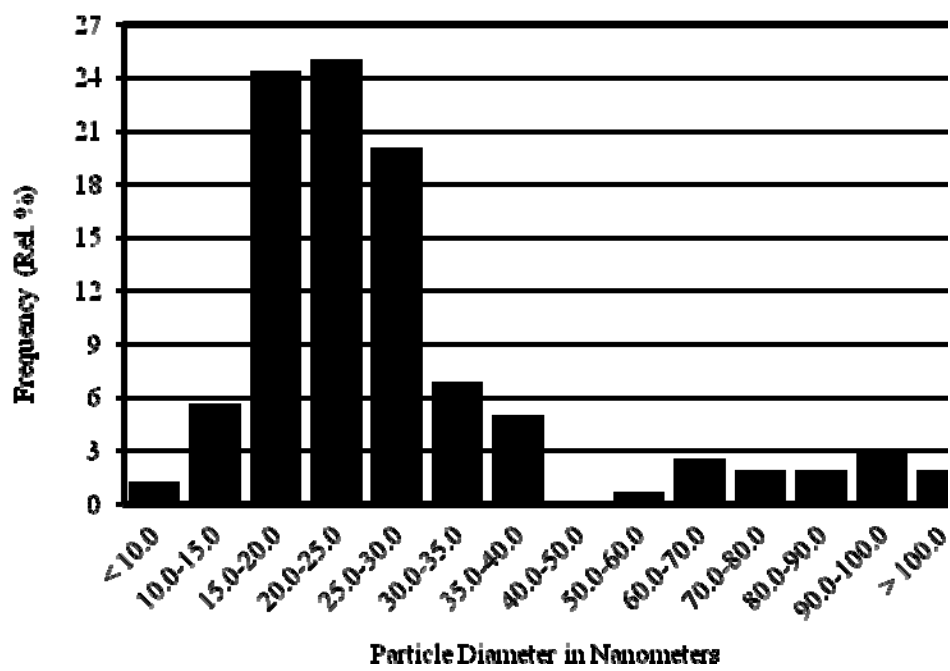


Figure 2. Cell viability (MTT assay) of human dermal fibroblasts exposed to different concentrations of ZnO NPs for 24 h. Data represented are mean±SD of three identical experiments made in three replicate. Statistically significant differences as compared to the controls. *p < 0.05 ** p < 0.001

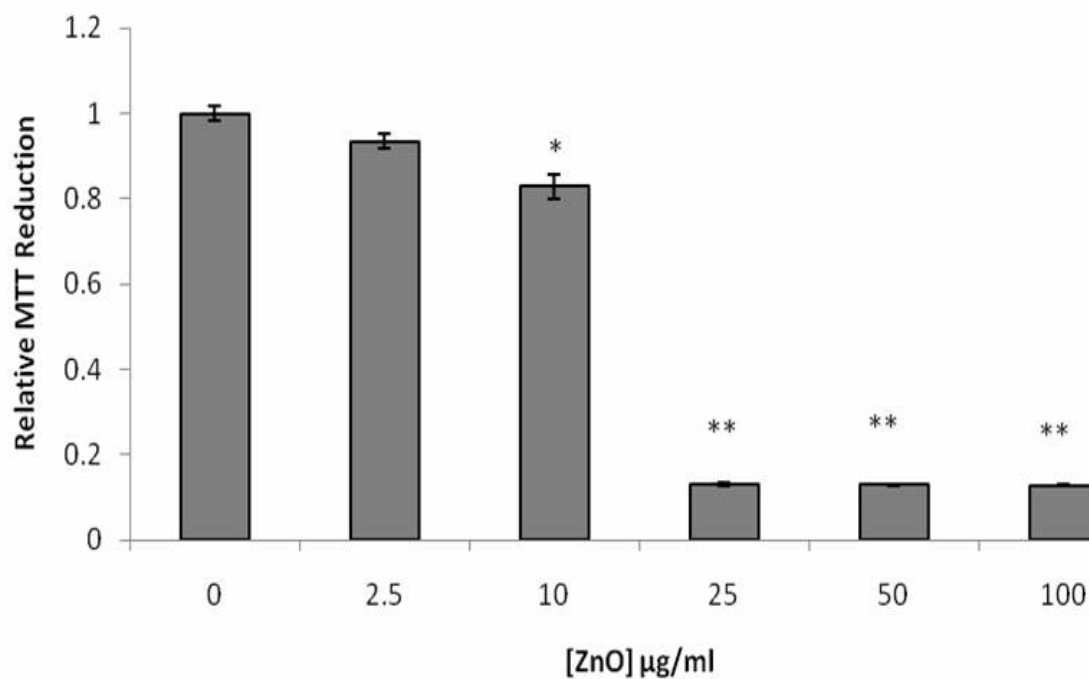


Figure 3. Morphology of human dermal fibroblasts exposed to ZnO NPs. (A) and (D) are control cells. (B) 50 $\mu\text{g/ml}$ for 4 h, (C) 100 $\mu\text{g/ml}$ for 4 h, (E) 50 $\mu\text{g/ml}$ for 24 h and (F) 100 $\mu\text{g/ml}$ for 24 hrs.

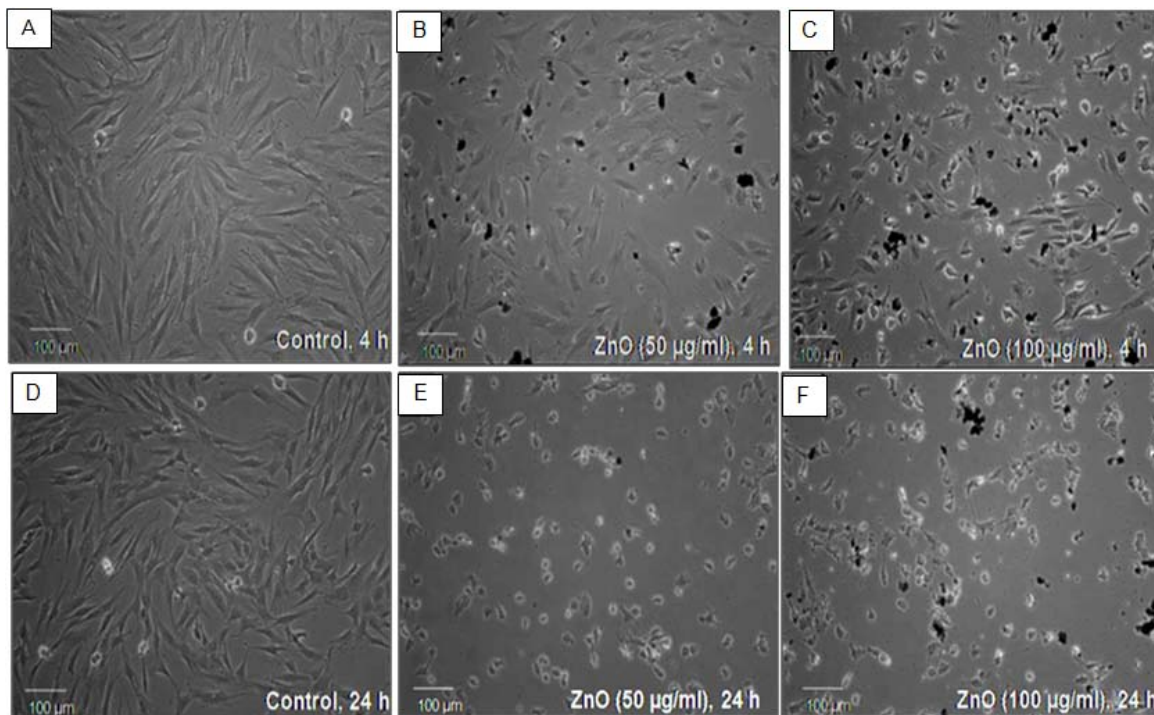


Figure 4. Annexin V staining result of control cells and 24 h after treated with 50 $\mu\text{g/ml}$ of ZnO NPs. Upper panel is untreated human dermal fibroblast cells staining with Annexin V. Lower panel is ZnO NPs treated human dermal fibroblast cells staining with Annexin V.

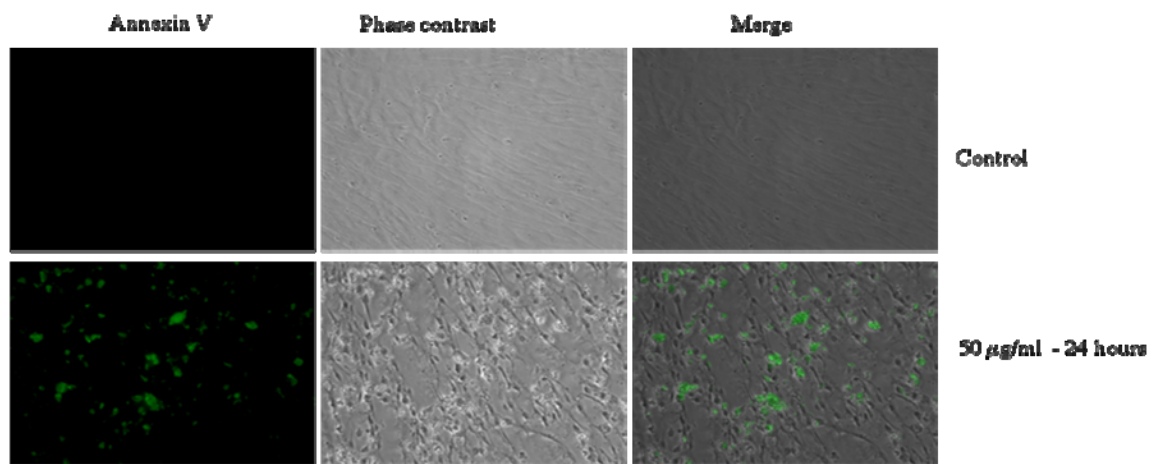


Figure 5. Effect of ZnO NPs on the expression level of p53 protein in human dermal fibroblast samples: (A) Lane 1-4 are 4 h after exposure (1) control, (2) 10 $\mu\text{g/ml}$, (3) 50 $\mu\text{g/ml}$ and (4) 100 $\mu\text{g/ml}$. (B) Lane 5-8 are 24 h after exposure (5) control, (6) 10 $\mu\text{g/ml}$, (7) 50 $\mu\text{g/ml}$ and (8) 100 $\mu\text{g/ml}$ (8). β -actin was used as loading control. The densitometry analysis data showed the significance level of protein expression. *Statistically significant difference as compared to the controls ($p < 0.05$ for each).

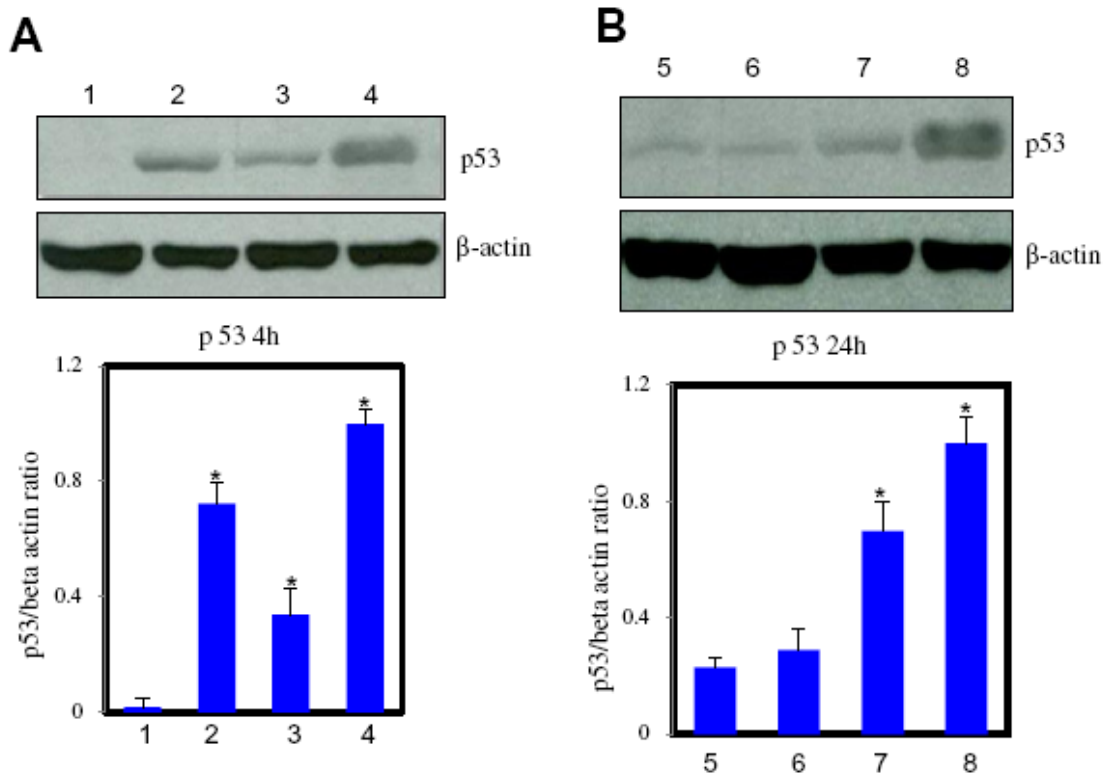


Figure 6. Effect of ZnO NPs on expression level of phosphor-p53 (Ser³³) in human dermal fibroblast cells. (A) Lane 1-4 are 4 h after exposure (1) control, (2) 10 µg/ml, (3) 50 µg/ml and (4) 100 µg/ml. (B) Lane 5-8 are 24 h after exposure (5) control, (6) 10 µg/ml, (7) 50 µg/ml and (8) 100 µg/ml. The densitometric analysis data showed the significance level of protein expression. *Statistically significant difference as compared to the controls (p<0.05 for each).

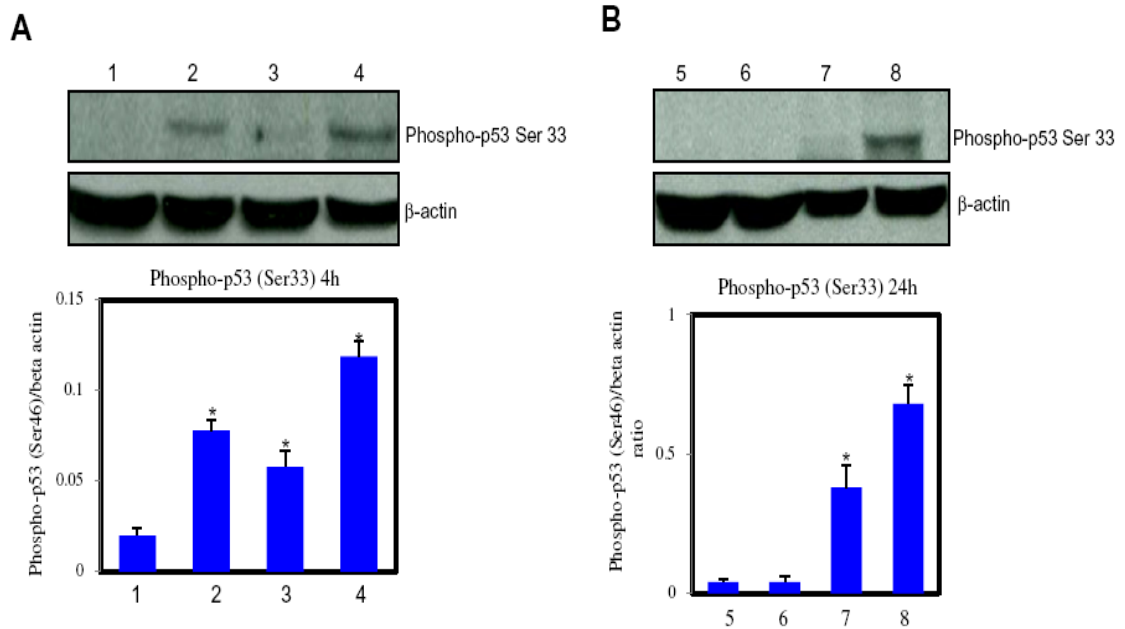


Figure 7. Effect of ZnO NPs on expression level of phosphor-p53 (Ser⁴⁶) in human dermal fibroblast cells. (A) Lane 1-4 are 4 h after exposure (1) control, (2) 10 $\mu\text{g/ml}$, (3) 50 $\mu\text{g/ml}$ and (4) 100 $\mu\text{g/ml}$. (B) Lane 5-8 are 24 h after exposure (5) control, (6) 10 $\mu\text{g/ml}$, (7) 50 $\mu\text{g/ml}$ and (8) 100 $\mu\text{g/ml}$. The desitometric analysis data showed the significance level of protein expression. *Statistically significant difference as compared to the controls ($p < 0.05$ for each).

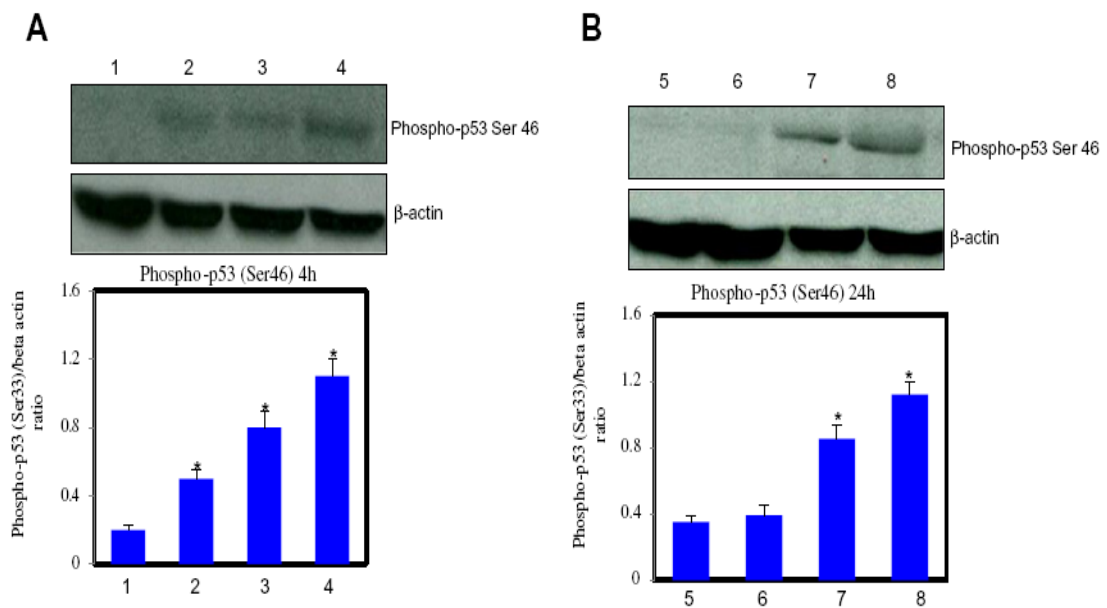
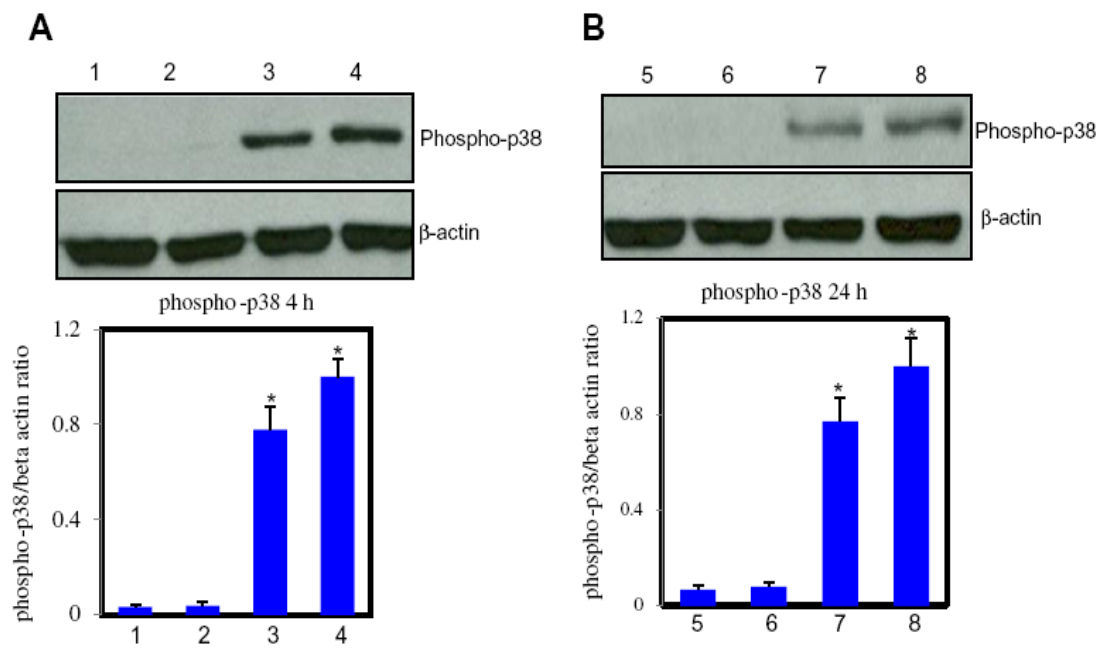


Figure 8. Effect of ZnO NPs on the expression level of p38 protein in human dermal fibroblast samples: (A) Lane 1-4 are 4 h after exposure (1) control, (2) 10 $\mu\text{g/ml}$, (3) 50 $\mu\text{g/ml}$ and (4) 100 $\mu\text{g/ml}$. (B) Lane 5-8 are 24 h after exposure (5) control, (6) 10 $\mu\text{g/ml}$, (7) 50 $\mu\text{g/ml}$ and (8) 100 $\mu\text{g/ml}$ (8). β -actin was used as loading control. The desitometric analysis data showed the significance level of protein expression. *Statistically significant difference as compared to the controls ($p < 0.05$ for each).



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