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Directing Human Embryonic Stem Cell Differentiation by Non-Viral Delivery of siRNA in 3D Culture

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Abstract

Human embryonic stem cells (hESCs) hold great potential as a resource for regenerative medicine. Before achieving therapeutic relevancy, methods must be developed to control stem cell differentiation. It is clear that stem cells can respond to genetic signals, such as those imparted by nucleic acids, to promote lineage-specific differentiation. Here we have developed an efficient system for delivering siRNA to hESCs in a 3D culture matrix using lipid-like materials. We show that non-viral siRNA delivery in a 3D scaffolds can efficiently knockdown 90% of GFP expression in GFP-hESCs. We further show that this system can be used as a platform for directing hESC differentiation. Through siRNA silencing of the KDR receptor gene, we achieve concurrent downregulation (60–90%) in genes representative of the endoderm germ layer and significant upregulation of genes representative of the mesoderm germ layer (27–90 fold). This demonstrates that siRNA can direct stem cell differentiation by blocking genes representative of one germ layer and also provides a particularly powerful means to isolate the endoderm germ layer from the mesoderm and ectoderm. This ability to inhibit endoderm germ layer differentiation could allow for improved control over hESC differentiation to desired cell types.

Keywords

siRNA delivery; gene therapy; human embryonic stem cells; differentiation; germ layers; KDR; 3D culture

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Introduction

Stem cells are a promising cell source for regenerative medicine, especially within the rapidly expanding field of tissue engineering. Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) are capable of many rounds of cell division and can differentiate into all of the cell types in the body[1, 2]. Natural differentiation starts in the embryo with the segregation of epiblast cells into the three germ layers, a process called gastrulation. This process results in a cell population containing all three germ layers. During this process, epiblast cells migrate through the primitive streak from the surface of the embryo to a more internal location. The cells that are left on the outer surface of the embryo will form the ectoderm, while those cells that migrate through the primitive streak will form the mesoderm and endoderm[3]. To isolate a specific germ layer to be used for regenerative purposes, protocols must be developed to control cell differentiation. However, in general, current differentiation protocols suffer from low transformation efficiency resulting in mixtures of mature and immature cell populations[4]. Moreover, while previous work has shown that derivation of ectoderm and mesendoderm can be achieved by supplementing the media with appropriate cytokines[1, 2] to date, segregation of the mesoderm and endoderm germ layers is not easily achieved, and requires elaborate and long term differentiation protocols.

One approach towards directing differentiation in hESCs is through gene silencing. As more genes are implicated in lineage-specific commitment, the ability to downregulate an entire germ layer, leading to a more pure population of differentiated cells, becomes particularly attractive. In addition, effective delivery of siRNA for gene silencing can lead to an increased understanding of the role of specific genes in the natural development of cell differentiation. We hypothesized that downregulation of specific genes can manipulate signaling pathways to either induce the growth of a specific sub-population of cells or interfere with the formation of an undesired population. Both circumstances result in the enrichment of a desired cell type. To test our hypothesis we introduced a combination of 3D tissue engineered matrices and siRNA delivery to develop a physiologically relevant siRNA delivery system for efficient transfection of hESCs (60–90% silencing). We then use the platform to explore the scenario of directing hESC differentiation towards a specific lineage by knocking down Kinase Insert Domain Receptor (KDR, also known as Vascular Endothelial Growth Factor Receptor 2 [VEGFR2], or Fetal Liver Kinase-1, [FLK-1]). Earlier work focused on regulation of gene expression by RNAi in hESCs has been demonstrated for genes such as OCT4[5–12], NANOG[9, 10] and SOX2[10] which are predominant during the undifferentiated state; however, in these cases differentiation was not directed towards specific germ layers. Instead an increase in expression of trophoblasts and endodermal genes was detected. To date, no work has investigated genes, such as KDR, that are not naturally downregulated during the differentiation process.

Thus far, delivery of siRNA has relied either on stable transfection using viral means[10, 11] or transient transfection using commercially available reagents (Lipofectamin, HiperFect) [5–9, 13, 14]. Recently, nucleofection[15] and siRNA conjugated to peptide RNA-binding transduction domains was reported to facilitate siRNA uptake in a variety of primary cell lines including hESCs[12]; however, efficiency in hESCs was not reported. *In vitro* experiments involving RNAi to date have been performed in a 2D environment where the cells are seeded on a surface and the transfection reagents are added into the media. It is well established that the 2D environment is different from the *in vivo* 3D cellular microenvironment[16–18]. Here we developed a 3D siRNA environment that facilitates high levels of up to 90% transfection. The scaffolds function as a substrate for cell infiltration, organization, and differentiation, while siRNA therapy provides the essential cues for differentiation. These were modified using lipid-like materials, known as lipidoids, that have

shown utility in siRNA delivery[19]. Previous work had identified lipidoids that provide for efficient siRNA delivery both *in vitro* and *in vivo*[19]. One material in particular, $98N_{12}(5)$ can self-assemble into nanoparticles and form complexes with siRNA based on electrostatic interaction (Supporting Data 1). These nanocomplexes can be internalized by cells through endocytosis and released from the endosome to the cytoplasma leading to the intracellular gene delivery. Interestingly, the 3D siRNA delivery methods developed here are significantly more efficacious than similar 2D growth environments. Using this 3D system, we explore the effects of specific gene silencing on hESC differentiation and discover that a single siRNA, targeting KDR, can prevent the differentiation of an entire germ layer, the endoderm.

Materials and Methods

Cell culture

hESC (H9, Wicell) and GFP expressing hESC (GFP-ES, EF1α-EmGFP-BG01v, Invitrogen) were grown on mouse embryonic fibroblasts (Chemicon) in hESC media (80 % knock out DMEM, 20 % knock out serum, 1 mM glutamine, 0.1mM beta mercaptoethanol, 1 % non essential amino acids and 5ng/ml b-FGF), as previously described[18]. 10μg/mL Hygromycin B (Invitrogen) was added to growth media for GFP-ES to prevent losing the GFP expressing cassette. Induction of differentiation was done by removing the cells from the feeder layer and transferring to petri dishes. This allowed formation of embryoid bodies (EBs) and induction of cell differentiations. EB formation was started in 15cm plates in suspension, and approximately 3×10^6 cells were generated in each plate. Cells were allowed to incubate in the presence of EB media (80 % knock out DMEM, 20 % knock out serum, 1 mM glutamine, 0.1 mM beta mercaptoethanol and 1 % non essential amino acids). Media was changed every 2 days.

3D siRNA delivery

Human ESCs or 8 day old EBs were dissociated through trypsinization and seeded onto 3D scaffolds (about 1×10^6 per $5 \times 5 \times 1$ mm³scaffold). During the seeding procedure, hESCs were mixed with either matrigel (Growth factor reduced matrigel, BD) solution, matrigel containing lipidoid-siRNA complexes or matrigel containing free siRNA. The cell/matrigel solution was loaded onto the scaffolds and allowed to solidify at 37°C, trapping the cells (and particles) inside the scaffolds. Scaffolds seeded with hESC (or 8 day old EBs) were grown in 2mL medium under gentile shaking.

Scaffold fabrication

Binary poly(l-lactic acid) and poly(lactic co-glycoilc acid) (PLLA/PLGA) scaffolds were prepared by dissolving the polymers at 50/50 weight ratios in chloroform to yield 5 % (w/v) polymer solution. NaCl grains were sieved to isolate salt grains of 212–500 μm in diameter. Then 0.24 mL of 50/50 PLLA/PLGA solution was added to Teflon molds containing 0.4 g of sieved NaCl. Following overnight solvent evaporation, rigid scaffolds containing salt grains were formed. Salt was leached out by immersion in deionized water for 6 hours to produce porous scaffolds. The same scaffolds were then lyophilized to remove any trace of solvent.

Lipidoid-siRNA complex formation

$98N_{12}(5)$ lipidoid was synthesized by Michael addition of triethylenetetramine and n-dodecylacrylamide [19]. GFP-siRNA (siGFP) and KDR-siRNA (siKDR) were complexed with $98N_{12}(5)$ (Supporting Data 1), in a sodium acetate (NaAc) buffer (25 Mm, PH5.2) for

15 min. Lipidoid/RNA weight ratio (3:1, 5:1) and RNA dosing (1, 0.5, 0.25, 0.125, 0.025μg) was varied.

Real time PCR

Following 3 or 10 days of culturing, hESC seeded scaffolds were homogenized using iron beads and Mini Bead Beater[TM] (Biospec Products) and total RNA was extracted using RNEasy mini kit (Qiagen, Catsworth, CA). 100–500 ng of total isolated RNA was reverse transcribed with Super Script III First Strand (Invitrogen). Aliquots of cDNA equivalent to 90 ng of total RNA were amplified using standard procedures with Taq Polymerase and Step One Plus Real time PCR System (Applied Biosystems). Quantified gene expression values were normalized against GAPDH (or β-actin if GAPDH was knocked down). Normalized expression $= 2^{[-(Ctsample - CtGAPDH]}$. The mean minimal cycle threshold values (Ct) were calculated from quadruplicate reactions. After normalization, samples were plotted relative to expression in EB's that were grown in suspension for the same time. Human ESC differentiation to the three germ layers (endoderm, ectoderm and mesoderm) on different scaffolds was evaluated by expression of selected genes (summarized in Table 1).

Flow cytometry

Cells were harvested from seeded scaffolds by dispase (Invitrogen) treatment for 20 minutes under gentle shaking. GFP expression was measured using fluorescence-activated cell sorting (FACS LSR II HTS, Becton Dickinson, Franklin Lakes, NJ, USA). Propidium iodide (PI) staining was used to exclude dead cells from the analysis. For KDR knockdown analysis following cell harvesting from seeded scaffolds, dead cells were depleted by using Dead Cell Removal Kit (Miltenyi Biotec). Single cells were aliquoted (5 to 7×10^5 cells were used per condition) and stained with either isotype controls or KDR-specific antibodies. Gating and analysis was performed using FlowJo 6.3 software (TreeStar, Ashland, OR, USA).

Computational Analysis of Signaling Networks

The protein-protein interaction networks were constructed using the GeneGo platform[20]. Proteins of interest were imported and sub-networks around the proteins were created with an "auto-expand" algorithm. The auto-expand algorithm gradually expanded sub-networks around every object from the list of imported proteins. At every step, preference was given to objects with more connectivity to the initial object. Connectivity is directional (to and from the initial object), and both directions were considered separately. Expansion was halted when the subnetworks intersected, or when the overall network size reached some pre-established limit of 200. The networks were then exported to Cytoscape for further analysis and visualization of the final networks[21].

Results

siRNA knockdown in 3D hESC cultures

In order to create a 3D delivery environment for siRNA, cells were mixed with matrigel containing hESCs, or matrigel mixed with siRNA lipidoid nanoparticles (see experimental procedures). This mixture of matrigel, siRNA nanoparticles, and cells was then seeded onto a 3D porous scaffold, made from the biodegradable polymers PLLA and PLGA. hESCs expressing GFP linked to the EF1 gene were used as a model system. The EF1 gene is expressed in hESCs regardless of the differentiation state[22].

GFP expression of cell seeded scaffolds was examined by fluorescence microscopy. For scaffolds containing $98N_{12}(5)$-siGFP complexes, cells showed reduced GFP expression (Figure 1A). Importantly control scaffolds with lipidoids containing a scrambled siRNA

sequence or with siRNA but without the lipidoid carrier possessed substantially more GFP expressing cells (Figure 1A). GFP expression further decreased on the third day in $98N_{12}(5)$-siGFP containing scaffolds (Figure 1A). The state of differentiation of the hESCs did not affect GFP expression as significant GFP knockdown was observed for both hESC seeded scaffolds and 8 day old embryoid body (hEB) seeded scaffolds (Figure 1A).

To rule out cell death as the cause of the observed knockdown, live cell staining (CellTrace calcein red-orange. Invitrogen) revealed that non GFP expressing cells on the scaffold were alive (stained in red) as well as the remaining GFP expressing cells (yellow: co-localization of green and red). Therefore, the observed knockdown can be attributed to successful delivery of siGFP to GFP-hESCs and not to cell death (Figure 1B). Control images staining cell seeded scaffolds treated with lipidoid-scrambled siRNA or untreated show no changes (Figure 1B and Supporting Data 2).

To quantify the level of siRNA knockdown, GFP expression in EBs and hESCs seeded in scaffolds was assessed by quantitative PCR and flow cytometry following 3 days in culture (Figure 2). Flow cytometry analysis revealed nearly complete knockdown in GFP expression of EBs (98%) and hESCs (89%) seeded on $98N_{12}(5)$-siGFP containing scaffolds (Figure 2A and 2C). Cell viability measured by PI staining remained high (> 80%) (Figure 2B). No change in GFP expression was detected in control systems where cells were grown in scaffolds containing lipidoid-scrambled siRNA formulations or where free siGFP was added to the media (Figure 2A). Similar results were also observed for GFP gene expression measured by PCR. Nearly complete knockdown of GFP gene expression was observed in EBs (98%) and hESCs (87%) seeded on $98N_{12}(5)$-siGFP containing scaffolds, compared to cells seeded in scaffolds without siRNA complexes (Figure 2D). In contrast, no change in GFP gene expression was detected in any of the control systems.

Using flow cytometry we then compared the efficacy of delivering siRNA in our 3D scaffolds versus a 2D culture using the same lipid-like materials (Figure 2E). Keeping the same cell:siRNA ratios as in the 3D system (0.001ng siRNA/cell), $98N_{12}(5)$-siGFP was added to the media of pre-seeded 2D cultures of EBs and hESCs. No reduction in GFP expression was detected in these experiments. Doubling the amount of $98N_{12}(5)$-siGFP resulted in decreased GFP expression; however, this appeared to be due to toxicity as 72% of the cells were dead, staining positively with PI.

Using siRNA to control hESC differentiation

We hypothesized that the differentiation state of the hESCs could be directed by knocking down KDR, a type III tyrosine kinase receptor for vascular endothelial growth factor (VEGF). As seen in Figure 3, hESCs grown in a lipidoid-siKDR scaffold showed significant KDR knockdown (72%). We then examined the effect of KDR knockdown on the expression of genes representative of the three germ layers; mesoderm (FOXF1, MEOX1, BRACH), endoderm (KDR, SOX17, MIXL1, GSC, FOXA2) and ectoderm (SOX1, ZIC1). Control cells, grown on a scaffold without lipidoid-siRNA complexes, spontaneously differentiated into all three germ layers, as observed by the expression of the above mentioned genes representing the germ layers.

Remarkably, the reduction in KDR expression led to significant changes in hESC differentiation (Figure 3). All examined mesodermal representative markers were significantly upregulated (FOXF1 (increased expression by 27 fold), MEOX1 (43 fold) and BRACH (92 fold). Expression of ectodermal representative markers were slightly upregulated (SOX1 (increased expression by 5 fold and ZIC1 by 8 fold). On the other hand, all examined markers representative of endodermal differentiation were downregulated (SOX17 (reduced expression by 65%), GSC (96%), MIXl1 (98%) and FOXA2 (82%))

(Figure 3). No reduction in expression of any of these examined genes was observed in control hESCs seeded on scaffolds containing free siKDR (no lipidoid) (Figure 3). As anticipated, 3D delivery of scrambled siRNA via lipidoid encapsulation did not result in downregulation of KDR or any of the other examined genes representative of the three germ layers (Figure 3). To further validate the reproducibility of 3D siRNA delivery, we examined delivery of GAPDH siRNA. Similarly, hESC seeded scaffolds containing lipidoid-siGAPDH showed knockdown of GAPDH (65%) without any change of the levels of any germ layer genes (Figure 3).

To understand the relationship between KDR knockdown and the downregulation of endoderm specific transcription factors (SOX17, FOXA2, MIXl1 and GSC), and the upregulation of mesoderm specific transcription factors, we constructed a protein-protein interaction network that connected the undifferentiated cell state[23] (signature genes expressed include CD24, CD9, GATA3, LN28A, NANOG, PODXL, PO5F1, SPI1, TERF1, KIT, PROM1, TIE2, GALA, KLF1 and KDR) and a differentiated state that leads to the expression of the observed genotype (genes expressed by different germ layers). We created two separate protein-protein interaction networks, one that includes KDR (initial network) and another where KDR is virtually knocked out. We identified this virtual knockout network by removing the KDR node from the initial network along with its interactors but maintained the network connectivity. We then subtracted from the KDR-knockout network the initial network and kept the remaining connected nodes. That is, we identified the proteins that are uniquely expressed during the virtual KDR knockout. Analyzing the connectivity of the reduced network led to identification of a predominant gene, androgen receptor (AR), which most likely affects gene expression (Figure 4A). Interestingly, in the subset of the extracellular proteins at this reduced network we can identify that integrins, predominantly α5β1, α4β1, and αvβ3, remain present in the network after knocking out KDR.

Next we proceeded to test the validity of the protein map. Based on the map, we expect the androgen receptor to affect the expression of the mesoderm, endoderm and ectoderm germ layer genes (Figure 4B). To test this hypothesis we grew cells on scaffolds containing lipidoid-siAR (siRNA targeting the androgen receptor) complexes. As predicted, this led to a decrease in the expression of endoderm representative genes and upregulation of the mesoderm and ectoderm genes, similar to the effect of the KDR knockdown. The levels of KDR (a mesoderm-specific gene) in the AR siRNA knockdown followed the trend of the rest of the mesoderm genes with significant increase in its expression.

Next we examined the significance of integrin signaling in the germ layer gene expression. Because several integrins were implicated in this pathway, it is technically challenging to downregulate integrins using siRNA. In this case we would need a different RNA sequence for each of the nine different integrins. Instead we chose a synthetic peptide Gly-Arg-Gly-Asp-Ser (GRGDS) because of its history blocking multiple integrin interactions [24–26].

This RGD-based peptide antagonizes integrin binding to the extracellular matrix (ECM) epitopes. In this experiment, hESCs were seeded onto a lipidoid-siKDR scaffold containing GRGDS to block integrin binding. Surprisingly, blocking integrins with the RGD peptide in parallel with siRNA delivery had no result in the KDR or any other germ line gene expression (Figure 4c). The gene expression in this case was similar to the control experiments where no lipidoids-siRNA complexes are delivered to the cells. We hypothesized that blocking the integrins on the surface of the hESCs significantly affects the siRNA delivery in the cells. In order to further evaluate our hypothesis, we examined the delivery of $98N_{12}(5)$-siGFP to GFP-hESCs in the presence of RGD peptide. No gene knockdown was observed by FACS analysis (Supporting Data 3).

Discussion

Here we have developed a 3D system for delivering siRNA to hESCs. We show that non-viral siRNA delivery in a 3D scaffolds can efficiently knockdown 90% of GFP expression in GFP-hESCs (Figure 1 and 2). The loss of GFP expression was attributed to actual siGFP mediated knockdown and not to cell death, based on live cells staining (Figure 1B) and FACS analysis of dead cell (Figure 2B). We further show that this system can be used as a platform for directing hESC differentiation to specific cell types. By down regulating a single gene (KDR) we were able to block hESC differentiation towards the endoderm linage as evident by 60–90% down regulation of genes representative of endoderm differentiation (Figure 3). The KDR knockdown also resulted in a dramatic increase in genes representative of the mesoderm germ layer. KDR levels are significant and relatively unchanged during the maturation of hESCs[23]. Therefore, we attribute changes in the differentiation of hESC following KDR knockdown could be attributed to the knockdown of this gene and not to the process of natural differentiation. To study the signallin events occurring during KDR knockdown, we constructed a protein-protein interaction network that describes the interactions of proteins, leading to the expression of the genes of interest. Such an approach can provide insight into pathways regulating the expression of proteins during stem cell differentiation[27, 28]. By reverse engineering we identified all possible combinations of proteins that interact with each other, creating pathways that link the expression of the aforementioned signature genes of the undifferentiated cell state to those expressed by the differentiated germ layers. Such networks can be constructed by mining information from various protein-protein interaction databases. These databases contain information on protein interactions from in vitro experiments or theoretical predictions, and describe physical or biochemical protein interactions, as well as transgene regulation patterns. Once a predominant signaling network was established describing all the possible combinations of protein linkages that affect the regulation of the transcription factors, we hypothesized that the state of the network can be perturbed by virtually knocking out KDR and reconstituting the initially identified network without KDR. Identifying the unique proteins in the KDR knockout network helped us develop a hypothetical roadmap describing the function of KDR during cell differentiation. Given the significance of the AR in the identified network connectivity we hypothesized that along with KDR, the AR is important for the expression profiles of the gastrulation specific transcription factors, the downregulation of the endoderm genes, and the upregulation of the mesoderm and ectoderm genes. In the later case, integrins seem to play an important role, according to the interaction network. Further use of our delivery platform confirmed the importance of AR (Figure 4).

AR is often studied in prostate cancer cell lines due to its role in the metastatic stage of this cancer type. In these cell lines AR has been linked to increased cancer cell growth and survival (through activation of the phosphatidylinositol 3-kinase(PI3K)/Akt pathway[29]) as well as increased cancer cell migration (through Activin A promotion[30]). In general, the PI3K/Akt pathway mediates a wide spectrum of cellular functions, including cell growth and transformation, differentiation, motility, and cell survival[31]. Recently, it has been shown that hESC differentiation to definitive endoderm requires concurrent Activin A stimulation and inhibition of the PI3K pathway[32]. Given the similarities between hESCs and cancer cells in their migratory and proliferative behavior, it is possible that AR activates the PI3K pathway in hESCs as well, leading to suppression of endodermal differentiation.

KDR is the receptor for VEGF, an essential component in blood vessel vasculogenesis and angiogenesis[33] It regulates vascular endothelial proliferation, migration, survival and increased permeability through intracellular signaling cascades, including the PI3K pathway (reviewed in[34] and [35]). In the embryo, the cardiovascular system is the first organ system to develop. KDR and VEGF are crucial mediators of this organ development[36].

KDR expression is observed early in embryonic development (at E7.0) and subsequently is restricted to endothelial precursor cells and hematopoietic cells[37]. The significant role of this receptor is underlined by fact that mice deficient in this molecule die at E8.5–9.5 due to defective development of blood islands, endothelial cells and haematopoietic cells[38]. Thus it has been difficult to study long term effects of KDR deficiency on organogenesis.

There are reasons to believe that endodermal development is linked to KDR; VEGF signaling is required first during the initial differentiation of the dorsal endoderm and second in the coalescence of the anterior endoderm at the midline to form the primitive gut tube and associated organs. It is known that disruptions of this process result in deformities such as foregut duplication and bilateral liver and pancreas development[39]. This is compatible with our studies finding that KDR knockdown results in downregulation of all examined endodermal representative genes.

Our data complements recent evidence suggesting the fundamental role of blood vessels providing developmental cues to organs and cellular differentiation. Early liver development was shown to depend on the presence of blood vessels even in vitro, in the absence of blood flow[40, 41]. A similar dependence of pancreas and kidney development on endothelial cells was reported[42]. Endothelial progenitor cells were associated with emerging buds of embryonic lung and the nascent glandular portion of the stomach[43]. Our gene level observations further validate the conclusions drawn from these studies performed at the tissue-level.

Conclusions

We believe that the efficient, 3D transfection/culture methods developed here provide a framework for the study of *in vivo* cell and tissue development during embryogenesis and the corresponding signaling pathways. These types of studies can shed light on the cues that drive stem cell fate, which can then be incorporated into the design of future 3D microenvironments to optimize and facilitate tissue repair and regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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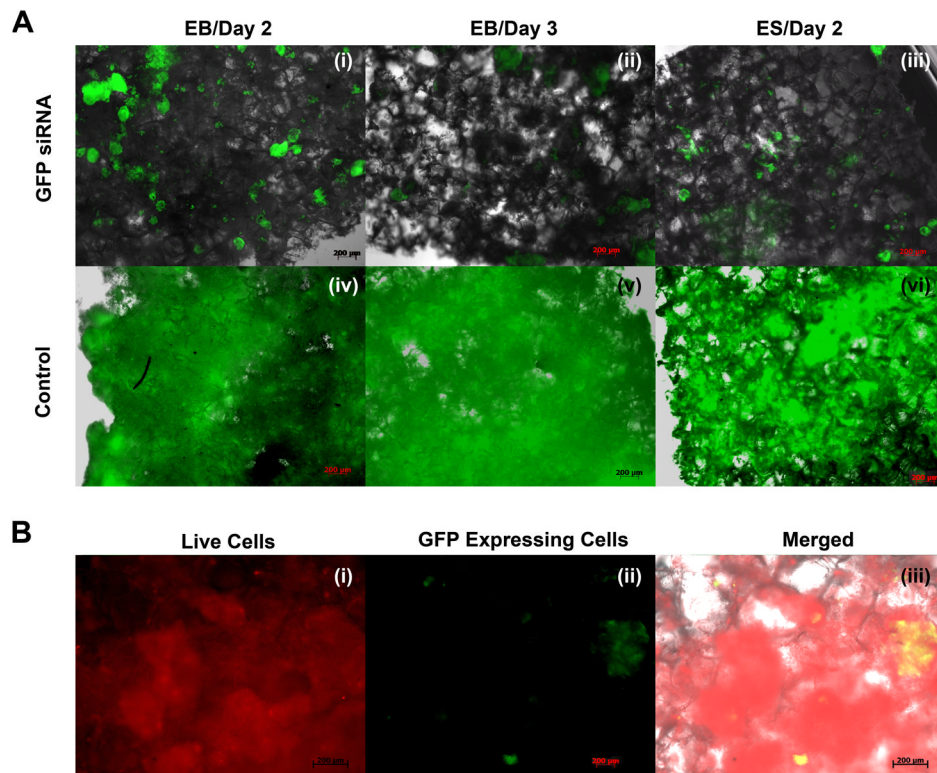
Figure 1.
Knockdown of GFP expression in GFP-hESC and GFP-hEB seeded scaffolds: A) Bright filed micrographs of GFP-hESC and GFP-hEB following 2 days and 3 days post seeding on scaffolds containing lipidoid- siGFP, lipid-siScrambled (vi) or untreated (iv, v). B) Live cell staining of GFP-hESC seeded scaffolds containing lipidoid-siGFP complexes. Live cells are stained in red, GFP-hESCs in green and colocalized live GFP-hESCs cells in yellow.
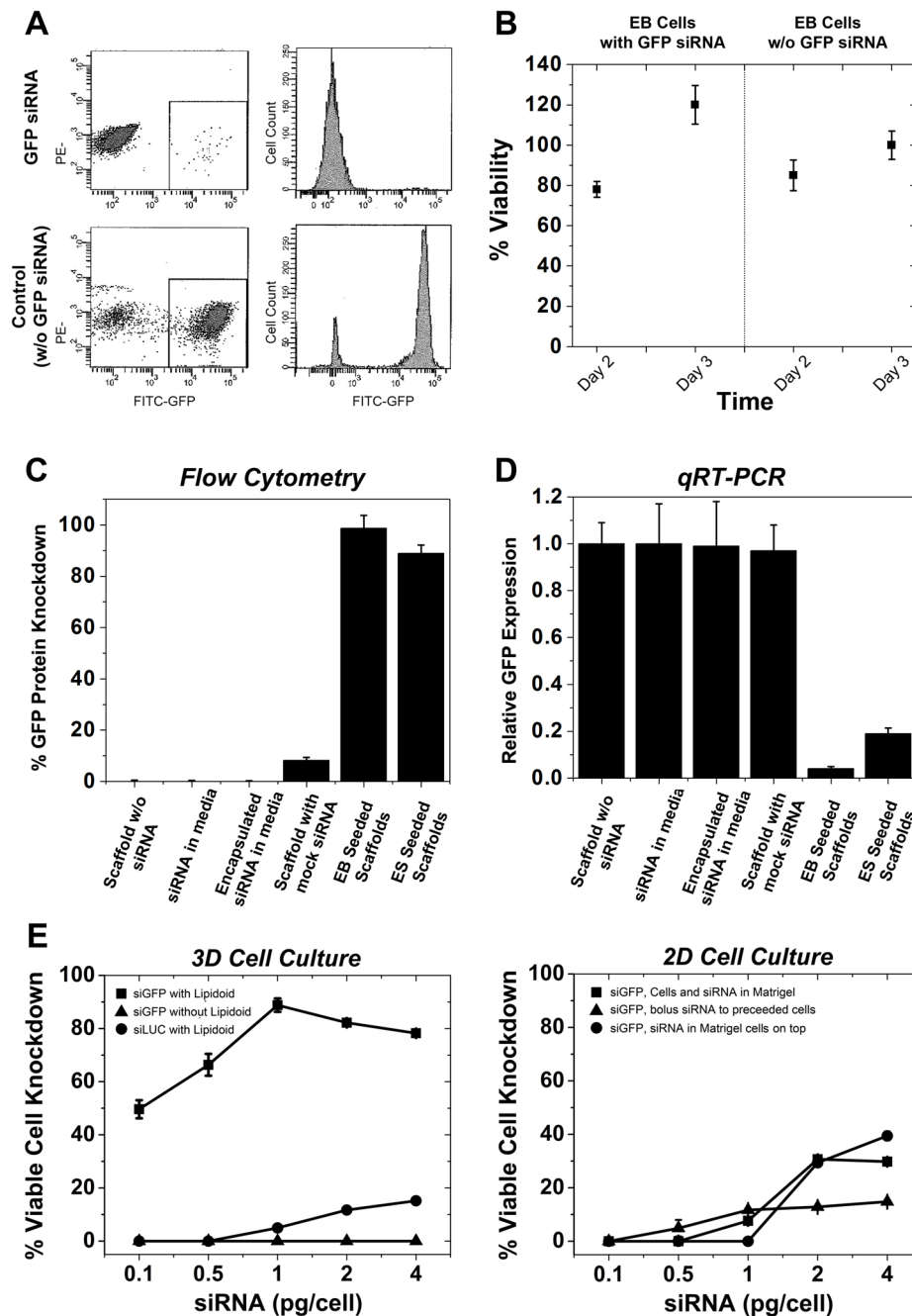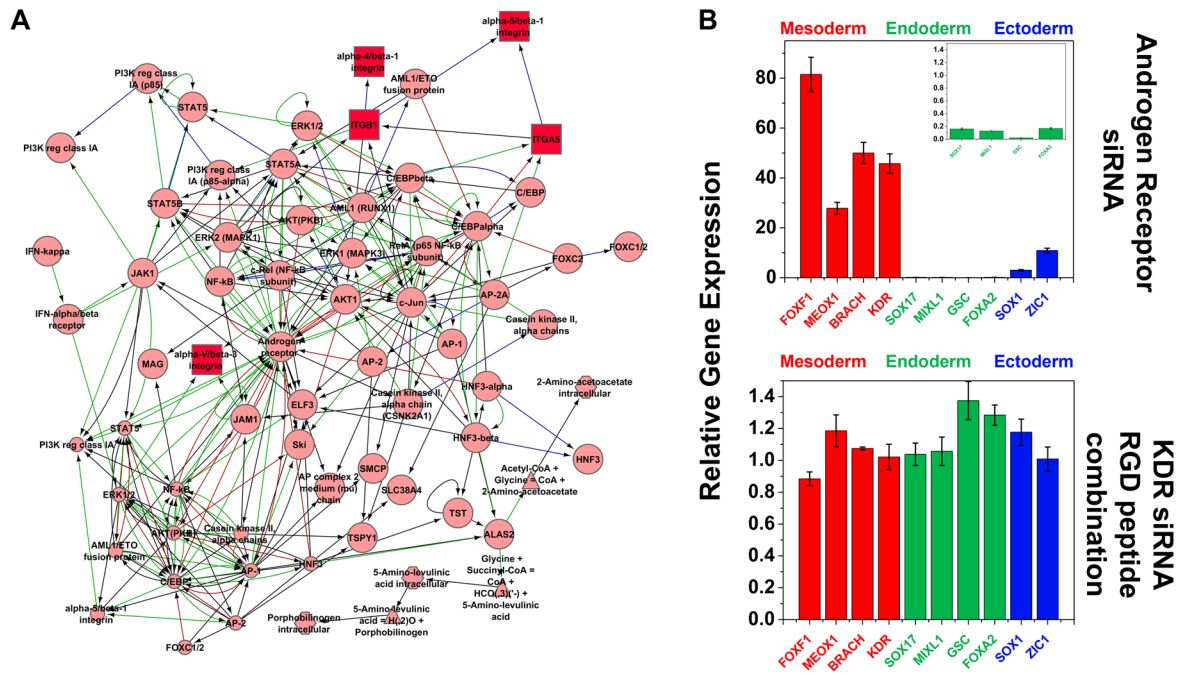
Figure 2.
Quantitative analysis of GFP knockdown in GFP-hESC and GFP-hEB seeded scaffolds: A) FACS analysis of GFP-hESCs grown for three days on 3D scaffolds containing lipidiod-siGFP and untreated. B) hEB viability measured by PI staining after growing in scaffolds for 2 and 3 days;. C) GFP expression of hESCs and hEBs measured by FACS. D) GFP expression of hESCs and hEBs measured by rt-qPCR normalized to untreated hESCs grown on 3D scaffolds. E) Dose response of hESCs treated with lipidoid-siGFP in 2D and 3D.
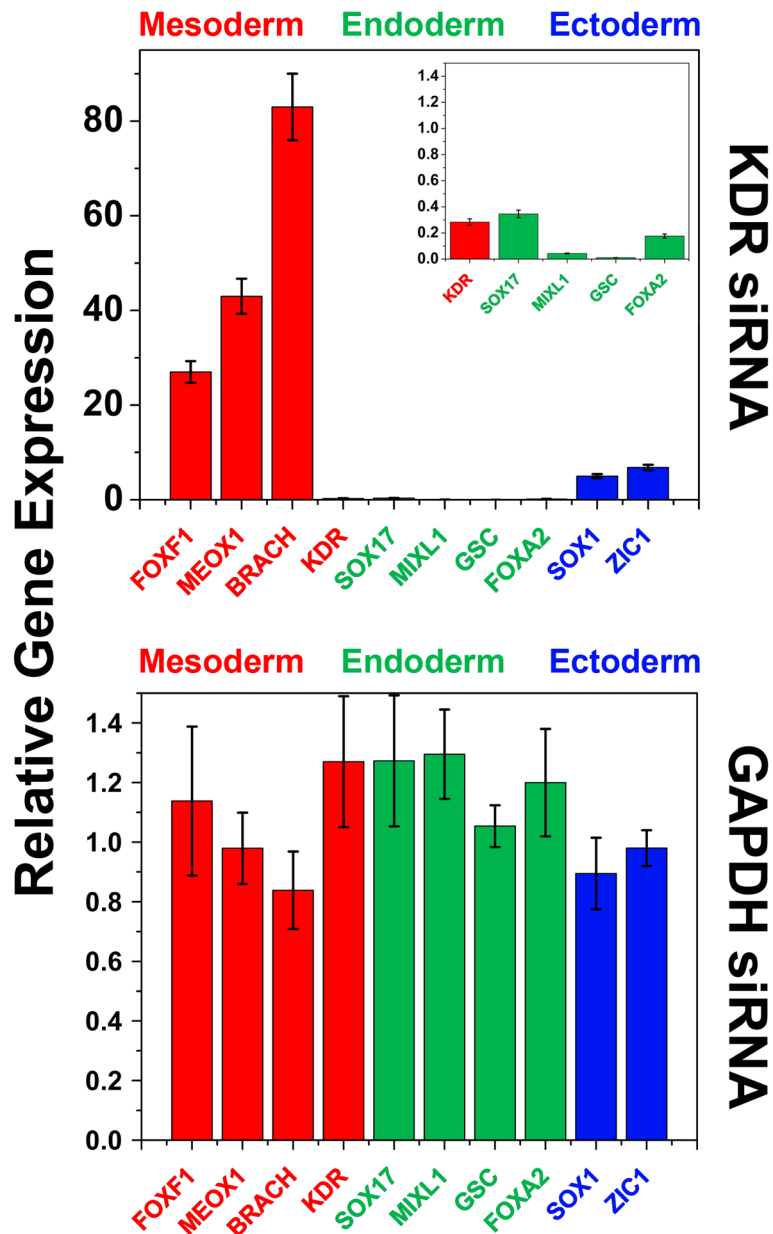
Figure 3.
RT-qPCR analysis of hESCs differentiation to the three germ layers following 3D delivery of (a) lipidoid-siKDR, and (b) lipidoid-siGAPDH. The siKDR upregulates the mesodermal genes with concomitant downregulation of the endoderm specific genes. Gene expression was normalized to untreated hESCs grown on 3D scaffolds.

Figure 4.
A) Protein-protein interaction network that leads to the expression of the gastrulation specific genes during KDR siRNA knockout. We then constructed a KDR reduced network introducing all genes that can preserve the connectivity of the network. These genes were identified as responsible for the expression of the gastrulation specific transcription factors once KDR is absent. B) Role of androgen receptor (AR): RT-qPCR of hESC gene expression following 3D delivery of lipidoid-siAR. C) Role of integrins: RT-qPCR of hESC gene expression following 3D delivery of lipidoid-siKDR in the presence of synthetic RGD peptide blocking integrins.