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Nanoparticle-mediated siRNA delivery assessed in a 3D co-culture model simulating prostate cancer bone metastasis

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ABSTRACT

siRNA has emerged as a potential therapeutic for the treatment of prostate cancer but effective delivery remains a major barrier to its clinical application. This study aimed to develop and characterise a 3D *in vitro* co-culture model to simulate prostate cancer bone metastasis and to assess the ability of the model to investigate nanoparticle-mediated siRNA delivery and gene knockdown. PC3 or LNCaP prostate cancer cells were co-cultured with hFOB 1.19 osteoblast cells in 2D on plastic tissue culture plates and in 3D on collagen scaffolds mimicking the bone microenvironment. To characterise the co-culture model, cell proliferation, enzyme secretion and the utility of two different gene delivery vectors to mediate siRNA uptake and gene knockdown were assessed. Cell proliferation was reduced by ~50% by day 7 in the co-culture system relative to monoculture (PC3 and LNCaP co-cultures, in 2D and 3D) and an enhanced level of MMP9 (a marker of bone metastasis) was secreted into the media (1.2–4-fold increase depending on the co-culture system). A cationic cyclodextrin gene delivery vector proved significantly less toxic in the co-culture system relative to the commercially available vector Lipofectamine 2000®. In addition, knockdown of both the GAPDH gene (minimum 15%) and RelA subunit of the NF-κB transcription factor (minimum 20%) was achieved in 2D and 3D cell co-cultures. Results indicate that the prostate cancer-osteoblast *in vitro* co-culture model was more physiologically relevant *vs* the monoculture. This model has the potential to help improve the design and efficacy of gene delivery formulations, to more accurately predict *in vivo* performance and, therefore, to reduce the risk of product failure in late-stage clinical development.

1. Introduction

Treatments for early stage prostate cancer are largely effective; however, if the disease progresses to a stage referred to as 'castrate resistant prostate cancer' it is almost always fatal (Valenca et al., 2015) and remains a significant cause of cancer-related death in men (Siegel et al., 2016). The most common site for the development of prostate cancer metastasis is in the bone (Bubendorf et al., 2000) and skeletal involvement correlates with a high level of patient mortality (Sathiakumar et al., 2011). Hence, improved agents for the treatment of prostate cancer that has spread to the bone are urgently required. Phase II clinical trials assessing new oncology products for the treatment of prostate cancer have a poor success rate, reported to be as low as 21% (Hay et al., 2014). Therefore, more effective biopharmaceutical screening tools to help identify lead candidates at an early stage of development are required.

In the pre-clinical development stage, current methods used to evaluate potential new cancer therapies largely rely on cancer cells cultured as monolayers on plastic tissue culture plates. These systems lack cell–cell and cell-matrix interactions and are thus not representative of *in vivo* cell growth (Ivanov et al., 2015). In

Abbreviations: DHT, dehydrothermal; MMP9, matrix metalloproteinase 9; MTT, 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; nHA, nanohydroxyapatite; PLK1, polio-like kinase 1; siRNA, small interfering RNA.

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contrast, by culturing cells in 3D in a physiologically-relevant environment, it is hoped that improved correlation between *in vitro* results and *in vivo* testing can be obtained, thus reducing the risk of expensive late-stage product failures during clinical development (Fitzgerald et al., 2015b). Such a physiologically relevant *in vitro* model offers the potential to investigate the performance and mechanism of action of novel treatments and drug delivery systems (Ivanov et al., 2015).

Small interfering RNA (siRNA) is a method of RNA interference which regulates the expression of specific genes and has shown promising *in vivo* results in animals for the treatment of prostate cancer (Guo et al., 2013). A major barrier to the clinical application of siRNA, however, remains the lack of a suitable delivery vector (Haussecker, 2012). Previously, our research group investigated the culture of prostate cancer cells in 3D on collagen-based scaffolds to mimic prostate cancer bone metastasis and subsequently used this model to assess the ability of siRNA nanoparticles to mediate gene knockdown (Fitzgerald et al., 2015a). Cells were cultured on scaffolds that simulated the bone microenvironment via the incorporation of collagen and nanohydroxyapatite (nHA). Collagen is the primary organic component of bone while the mineral matrix is mostly composed of hydroxyapatite crystals (Bayliss et al., 2012). nHA is recognised to have a composition that mimics the inorganic component of human bone (Hu et al., 2014). This model successfully facilitated prostate cancer cell growth in 3D and was capable of comparative evaluation of nanoparticulate gene therapeutics (Fitzgerald et al., 2015a). However, as it contained only one cell type, it did not permit cell–cell interaction as occurs *in vivo* between prostate cancer cells and cells of the bone tissue. Hence, one of the aims of this work was to further develop this model using a co-culture system which allows direct contact between prostate cancer and bone cells.

After prostate cancer cells have metastasized to the bone, they are thought to interact with osteoblasts through both soluble factors and physical contact (Shiirevnyamba et al., 2011). Hence, in this study, human cells derived from prostate cancer metastasis (PC3 and LNCaP cells) were cultured in direct contact with a human foetal osteoblast cell line (hFOB 1.19 cells) to better simulate the bone microenvironment. PC3 cells were originally derived from lumbar vertebrae metastasis and are known to be highly metastatic whereas LNCaP cells are derived from lymph node metastasis and have low metastatic potential (Aalinkeel et al., 2004; Webber et al., 1997). Cells were co-cultured in 2D on plastic tissue culture plates and also in 3D on the collagen-based scaffolds incorporating nHA (Fitzgerald et al., 2015a). The study thus aimed to determine conditions which would facilitate cell proliferation of both cell lines in co-culture and to establish an improved, more physiologically relevant *in vitro* 3D bone metastasis model. The co-culture model was characterised (cell proliferation, MMP9 enzyme secretion) and the potential of the model as a pre-clinical biopharmaceutical tool to evaluate the delivery and gene silencing capacity of siRNA nanocomplexes was investigated.

2. Materials/methods

2.1. Materials

All materials were purchased from Sigma-Aldrich (Wicklow, Ireland) unless otherwise stated. Negative control non-silencing siRNA (sense strand sequence 5′-UUC UCC GAA CGU GUC ACG U-3′) was purchased from Sigma. The same sequence modified with the fluorescent marker Alexa647 on the 3′ end of the sense strand was used for fluorescence experiments (Sigma). siRNA targeting the GAPDH endogenous housekeeping gene was purchased from Sigma (sense strand sequence 5′-CUG CCA AAU AUG AUG ACA-3′). RelA siRNA (sense strand sequence 5′-CCA UCA ACU AUG AUG AGU U-3′) was purchased from Sigma.

2.2. Cell culture

PC3 cells (human prostate cancer cell line, European Collection of Cell Cultures (ECACC), UK, originally derived from prostate cancer bone metastasis) were maintained in complete growth media consisting of RPMI-1640 medium, supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-Glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. LNCaP cells (human prostate cancer cell line, American Tissue Culture Collection (ATCC), USA, originally derived from prostate cancer lymph node metastasis) were maintained in complete growth media consisting of RPMI-1640 medium supplemented with 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-Glutamine, 10 mM HEPES, 7.2 mM D-glucose and 1 mM Sodium Pyruvate. LNCaP cells were cultured and seeded in Poly-D-Lysine (1×) coated tissue culture flasks and wells. Both cell lines were maintained at 5% CO_2 and 37 °C (Fitzgerald et al., 2015a). hFOB 1.19 cells (human foetal osteoblast cell line, American Tissue Culture Collection, U.S.A), originally derived from a spontaneous miscarriage and made immortal by transfection with a temperature sensitive variant of the large T-antigen from the Simian Virus (SV40) (Skogseth, 2010) were maintained in phenol-red DMEM/F12 supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 mM L-Glutamine (Shiirevnyamba et al., 2011). Osteoblast cells were maintained in 5% CO_2 at 34 °C.

2.3. Scaffold fabrication

Collagen-nHA scaffolds were prepared as previously reported (Fitzgerald et al., 2015a). Briefly, fibrillar collagen (Integra Life Science, Inc.) was blended with 0.05 M acetic acid to produce a collagen slurry. nHA particles were synthesized (Cunniffe et al., 2010; Curtin et al., 2012) and added to the collagen slurry during the blending process (2:1 ratio of nHA:collagen scaffold). Slurries were freeze-dried and all scaffolds were cross-linked and sterilised using a dehydrothermal (DHT) treatment (Haugh et al., 2009). Cylindrical scaffold samples (8 mm diameter, 4 mm height) were obtained using a biopsy punch and cross-linked with 14 mM *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride and 5.5 mM *N*-Hydroxysuccinimide (EDAC/NHS) in dH_2O (Murphy et al., 2010) for 2 h to improve the mechanical properties of the constructs.

2.4. Seeding of cells in monoculture/co-culture

For monoculture experiments, cells (PC3, LNCaP or hFOB 1.19 cells) were seeded at the required density and cultured in complete osteoblast growth media at 5% CO_2 and 37 °C. For co-culture experiments, prostate cancer cells (PC3 or LNCaP cells) were mixed with human osteoblasts (hFOB 1.19 cells) at a 1:1 ratio (Shiirevnyamba et al., 2011) and cultured in complete osteoblast growth media. Cells were maintained at 5% CO_2 and 37 °C. The exact seeding densities used in subsequent experiments are outlined in Table 1. The table details the number of either prostate cancer or osteoblast cells grown in monoculture experiments. For co-culture experiments, an equal number of prostate cancer and osteoblast cells were seeded (*i.e.* co-cultures had double the total number of cells relative to monoculture experiments).

For all cell work undertaken using LNCaP cells cultured in 2D and control hFOB 1.19 cell monocultures, tissue culture plates were pre-coated with Poly-D-Lysine (1×).

For seeding of cells in 3D on collagen-based scaffolds, cells (either monoculture or co-culture) were suspended in 50 μl media