**Brief Communication**

Initial Observations on the Effect of Medium Composition on the Differentiation of Murine Embryonic Stem Cells to Alveolar Type II Cells

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**ABSTRACT**

The pluripotency and high proliferative index of embryonic stem (ES) cells make them a good potential source of cells for tissue engineering purposes. We have shown that ES cells can be induced to differentiate *in vitro* into pulmonary epithelial cells (type II pneumocytes) using a serum-free medium designed for the maintenance of mature distal lung epithelial cells in culture (SAGM). However, the resulting cell cultures were heterogeneous. Our aim in this study was to attempt to increase pneumocyte yield and differentiation state by determining which medium components enhance the differentiation of pneumocytes and modifying the medium accordingly. Quantitative RT-PCR was used to measure changes in the expression of a type II pneumocyte-specific gene, surfactant protein C (SPC), in response to alterations in the cell culture medium. Results suggested that most individual SAGM growth factors were inhibitory for type II pneumocyte differentiation, with the largest increases in SPC expression (approximately threefold) being observed upon removal of retinoic acid and triiodothyronine. However, large standard deviations occurred between replicates, illustrating the highly variable nature of ES cell differentiation. Nevertheless, these observations represent an initial step towards achieving directed differentiation of pneumocytes from stem cells that could lead to their purification for tissue engineering purposes.

**INTRODUCTION**

The current mode of management of end-stage pulmonary disease is transplantation but the inadequate supply of donor lungs means that there is an acute need for alternative sources of a replacement gas-exchange unit. Tissue engineering is an emerging field of medicine and any programme aimed at producing functional lung to augment compromised breathing capacity will need to generate alveolar tissue. The epithelium of an alveolus is composed of two cell types; type I and II pneumocytes. Type I cells line the majority of the alveolus and perform the primary...
gas-exchange function. The more abundant but smaller type II cells secrete surfactant, a complex mixture of proteins and phospholipids, which lowers alveolar surface tension, facilitates gas exchange and prevents airway collapse. In peripheral lung injury, type II cells undergo proliferation and differentiation to the type I phenotype (Ott, 1997). Thus, type II cells are crucial to the normal regenerative process of the alveoli. In fact, type II cells are considered as putative alveolar stem cells (Ott, 1997). Primary type II pneumocytes have been isolated from the lungs of various mammals (Dobbs, 1990), but their yield and proliferation rate is low and they lose many of their characteristics a few days after being cultured (Sugahara et al., 1998). Embryonic stem (ES) cells are pluripotent cells with a high proliferative capacity derived from inner cell mass of the blastocyst (D’Amour and Gage, 2000; Odorico et al., 2001). Their derivation was first reported in 1981 from mice (Evans and Kaufman, 1981) and in 1988 from humans (Thomson et al., 1998). ES cells have the potential to produce any desired adult cell in an unlimited number, they could be an invaluable resource for tissue engineering purposes. We have shown previously that, by providing a medium designed for the growth and maintenance of mature alveolar cells (SAGM), it is possible to enhance the differentiation of type II alveolar cells from murine ES cells, although the resulting cell populations were heterogeneous (Ali et al., 2002). In this study, we aimed to increase the yield and differentiation state of pneumocytes from ES cells by modification of SAGM composition. SAGM is comprised of a basal medium, SABM, which is supplemented with a number of growth factors and hormones. A variety of strategies can be employed for the formulation of optimised culture media (Zimmerman et al., 2000); we chose the method of removing component medium factors in turn due to the ease with which this could be achieved in our culture system. Thus, a range of SAGM media was prepared in which these supplements were removed, one by one, allowing us to assess the contribution of each supplement to type II pneumocyte differentiation, without the loss of interactions between other SAGM supplements. As a rapid index of type II cell differentiation in each ES cell culture, quantitative RT-PCR for a type II cell-specific marker gene, surfactant protein C (SPC), was carried out.

**MATERIALS AND METHODS**

**Cell culture**

The murine embryonic stem cell line E14Tg2a (a kind gift of Prof. A. Smith, University of Edinburgh, UK) was routinely cultured on gelatin-coated tissue culture plates in high-glucose DMEM supplemented with 10% batch tested FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all Invitrogen, Paisley, UK), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, Dorset, UK) and 1000 U/mL leukaemia inhibitory factor (LIF; Chemicon, Temecula, CA). Cultures were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C.

E14Tg2a cells were differentiated as previously described (Ali et al., 2002). Briefly, embryoid bodies (EBs) were formed by suspension culture of small cell clusters (formed by limited trypsin digestion) in complete DMEM without LIF. At approximately 8–10 days of differentiation, EBs were transferred into gelatinised tissue culture flasks and allowed to differentiate in adherent culture up to 30 days. Cells were then transferred to six-well plates at a seeding density of 5 × 10⁴ cells/cm² and settled overnight in complete DMEM (without LIF). Cells were then further differentiated for 3 days, either in one of a variety of media modified from Small Airway Growth Medium (SAGM; Biowhittaker, Watersville, MD), or were left in DMEM. Complete SAGM consists of a basal medium (Small Airway Basal Medium, SABM) supplemented with 0.5 mg/mL bovine serum albumin, 5 μg/mL insulin, 10 μg/mL transferrin, 30 μg/mL bovine pituitary extract, 0.5 μg/mL epinephrine, 6.5 ng/mL triiodothyronine, 0.1 ng/mL retinoic acid, 0.5 μg/mL hydrocortisone and 0.5 ng/mL human epidermal growth factor. A range of media were prepared, each omitting a single supplement, in order to assess the contribution of each SAGM constituent to type II pneumocyte differentiation.

**Real-time reverse-transcription polymerase chain reaction**

RNA was extracted from differentiated E14Tg2a cells using TRIzol reagent (Invitrogen), quantified by spectrophotometry, and RNA integrity confirmed by denaturing agarose gel electrophoresis (data not shown). RNA was DNase treated using RQ1 RNase-free DNaseI (Promega, Madison, WI).
then 1 µg RNA was reverse transcribed into cDNA using the Thermoscript RT-PCR system (Invitrogen) and an oligo(dT)20 primer. 5% of the reverse transcription products were used as a template for real-time PCR analysis.

Real-time PCR was performed using a GeneAmp SDS 7500 thermal cycler and the SYBR Green PCR Core Reagents Kit (both Applied Biosystems, Foster City, CA). PCR reaction mixtures contained cDNA template, primers, 1 × SYBR Green PCR Buffer, 3 mM MgCl₂, 200 nM each of dATP, dCTP, dGTP and 400 nM dUTP, 0.75U AmpErase UNG in a final volume of 25 µL. Each PCR reaction was performed in triplicate to control for pipetting errors, and adult mouse lung cDNA positive controls and no-template negative controls were also included in each PCR run. Primers were designed using PrimerExpress software (Applied Biosystems) and primer concentrations were optimised for each primer pair according to the PCR kit protocol. Primer sequences, reaction concentrations and amplicon length are shown in Table 1. Thermal cycling parameters were: 50°C, 2 min; 95°C, 10 min; followed by 40 cycles of 95°C, 15 sec; 60°C, 1 min. Following the PCR reaction, baseline fluorescence was measured between cycle number 5 and two cycles prior to the initiation of PCR product amplification, to adjust each sample for background fluorescence. Threshold cycle (CT) values were then obtained by manually setting a threshold of fluorescence such that it intersected the logarithmic phase of every amplification plot within each PCR run. Dissociation curves were also generated at the end of each PCR reaction, in order to confirm the presence of a single specific reaction product in each positive sample.

Relative quantification of gene expression by the comparative CT method was chosen as the most appropriate method of analysis, allowing changes in SPC gene expression between ES cells treated with modified or complete SAGM medium to be compared directly. Two housekeeping genes with very different cellular functions were chosen for data normalisation, β-actin and GAPDH, to minimise the possibility of data distortion through up- or down-regulation of the housekeeping gene by modified cell culture media. For the ∆∆CT calculation to be valid, the amplification efficiency of each housekeeping gene PCR product must be approximately equal to that of the SPC product. This was tested by examining whether ∆CT varied with 1:2 serial dilution of the positive control adult mouse lung cDNA template. Results derived from differentiated ES cells were then analysed by the comparative Ct method according to the equation:

\[
\text{Amount of target (normalised to an endogenous reference gene and relative to the complete SAGM sample)} = 2^{-\Delta\Delta CT},
\]

where \(\Delta CT = CT_{\text{test sample}} - CT_{\text{complete SAGM}}\) and \(\Delta\Delta CT = CT_{\text{test sample}} - CT_{\text{housekeeping gene}}\).

Realtime RT-PCR results were analysed by one-way ANOVA with Dunnett’s multiple comparison post-test, using the Graphpad Prism statistical analysis software package (Graphpad Software Inc., CA).

## RESULTS

Primer validation for the comparative CT method of relative quantitative RT-PCR

For each pairing of SPC and housekeeping gene primers to be suitable for use with the comparative CT method of relative quantification, the plot of log2 relative input amount versus CT for SPC compared against each reference gene must have a gradient of 0.1 (manufacturer’s recommendation). The results are shown in Figure 1 and confirm the validity of both sets of primers in Table 1.

### Table 1. Quantitative RT-PCR Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon length</th>
<th>Reaction concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC rt F</td>
<td>ACCGCTGGTGGGACGCTTCCCA</td>
<td>88 bp</td>
<td>900 nM each</td>
</tr>
<tr>
<td>SPC rt R</td>
<td>TTTGGGAGGGGCTTTTCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-Act rt F</td>
<td>CAGAAGAGATTACCTGCTTCT</td>
<td>93 bp</td>
<td>300 nM each</td>
</tr>
<tr>
<td>B-Act rt R</td>
<td>GGACCCACCATCCACACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH rt F</td>
<td>TGTCGCTGGTCGGATCTGCA</td>
<td>77 bp</td>
<td>300 nM each</td>
</tr>
<tr>
<td>GAPDH rt R</td>
<td>CCTGCTCCACCCACCTCCTGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
primers for use with the comparative C_\text{T} method of analysis.

The effect of SAGM modification on SPC expression in differentiated mES cells

Normalization of SPC expression to both housekeeping genes yielded comparable results from the quantitative analysis, suggesting that the neither GAPDH nor β-actin gene expression was significantly affected by modulation of the SAGM culture medium (Fig. 2). Consistent with the results presented in our previous paper (Ali et al., 2002), treatment of the cells with complete SAGM medium following 30 days of differentiation in DMEM increased the expression of SPC mRNA in differentiated ES cell cultures by approximately 20-fold above those left in the basal differentiation medium (p < 0.01). Modification of the SAGM selection medium by sequential

FIG. 1. Each pairing of SPC and housekeeping gene primers was validated for use with the comparative C_\text{T} method of relative quantification. Quantitative PCR was performed in triplicate on serial 1:2 dilutions of adult mouse lung cDNA, and ΔC_\text{T} for each dilution (C_\text{T(SPC)} - C_\text{T(housekeeping)}) plotted against the log_2 of the relative template input. Error bars represent one standard deviation from the mean of the three replicates. Linear regression lines were plotted and the gradients shown to be less than 0.1.

FIG. 2. Differentiated ES cells exposed to variations on the cell culture medium SAGM were harvested for quantitative RT-PCR analysis of SPC expression by the comparative C_\text{T} method. Results for each SAGM variant were calibrated against complete SAGM, the mean of which was arbitrarily set at 1. Error bars represent one standard deviation from the mean of three replicates. White bars represent SPC expression normalised to the β-actin reference gene, black bars represent SPC expression normalised to GAPDH. BPE, bovine pituitary extract; T3, triiodothyronine; Ep, epinephrine; IGF, epidermal growth factor; HC, hydrocortisone; RA, retinoic acid; RF, “6-factors” (all supplements excluded except insulin, transferrin, BSA); trans, transferrin.
omission of a single growth factor or hormone supplement tended to upregulate SPC RNA expression, with the highest increase observed when either triiodothyronine (T3) or retinoic acid was removed (approximately threefold upregulation). However, statistical analysis of these results was confounded by the presence of a very high degree of variability between replicates. To attempt to reduce the observed variability, results that appeared to be outliers were repeated to rule out the possibility of PCR artefacts; however, essentially identical results were obtained from the second PCR run. Therefore, the variation in SPC RNA expression appeared to stem from an inherent biological variability of differentiated ES cells and not from erroneous quantification. Statistical analysis comparing SPC RNA expression in cells treated with complete SAGM against that from cells treated with each modified SAGM medium yielded a significant result (p < 0.05) for only the SAGM-T3 sample when SPC RNA expression was normalised to GAPDH. The apparent increases in SPC RNA expression caused by the exclusion of bovine pituitary extract, epinephrine, epithelial growth factor, retinoic acid, insulin and transferrin were rendered statistically insignificant as a consequence of the high standard deviations from the mean. Interestingly, only the omission of hydrocortisone indicated any SPC RNA expression from modification of the selection medium, again, this was not statistically significant.

DISCUSSION

Previously, we demonstrated that Small Airway Growth Medium (SAGM), a medium designed for the in vitro maintenance of distal lung-derived primary cells and cell lines, can be employed to enrich a population of differentiating murine embryonic stem cells for the type II alveolar cell phenotype (Ali et al., 2002). These findings were confirmed in this study, where real-time RT-PCR quantification of SPC gene expression revealed that cells cultured in SAGM following an extended period of differentiation exhibited 20-fold higher SPC gene expression than ES cells grown in the basic DMEM differentiation medium. However, the cell population that was derived was mixed. The identification of cell growth media which can target ES cell differentiation to desired cell lineages is a major topic in current stem cell research, as it would significantly increase the yield of the target cell population and thus enhance the efficiency of any regenerative therapy based on ex vivo stem cell differentiation. To this end, we attempted to augment further type II cell differentiation by altering the composition of regulatory growth factors, hormones and small molecules in SAGM. A wide variety of growth factors and hormones is known to play a significant role in lung branching morphogenesis and maturation of the various lung cell lineages from experiments in embryonic lung organ culture and transgenic mice. These include epidermal growth factor (EGF [Mirttinen et al., 1997; Warburton et al., 1992]), platelet-derived growth factor (PDGF; Souza et al., 1995), fibroblast growth factors (FGFs [Min et al., 1998; Stucky et al., 1996; Simonet et al., 1995; Whitsett et al., 2002]), transforming growth factor-β (TGFβ [Kaartinen et al., 1995; Serra et al., 1994; Zhou et al., 1996]), retinoids (Chazaud et al., 2003; Mendelsohn et al., 1994; Mollard et al., 2000), and glucocorticoids (Cole et al., 1995; Odom et al., 1988).

Modulation of the growth factor composition of SAGM was relatively easily achieved with this system, as complete SAGM is generated by adding a number of supplements to a basal medium, SABM. These supplements were each excluded from the complete medium one by one, such that the contribution of a single supplement to type II cell differentiation could be assessed without the loss of synergistic interactions between the signalling pathways of the other supplements. Our results suggest that the removal of each component of SAGM in turn increased type II cell differentiation. However, the combined removal of every supplement except insulin and transferrin did not increase SPC expression in an additive fashion, suggesting that a significant degree of synergism operates between these different signalling pathways. It is well known that gene expression is often regulated by the formation of large multi-protein transcription factor complexes on the gene promoter, in which each individual component contributes to the overall regulatory effect of the complex. Consequently, the regulatory activity of discrete transcription factors is often context dependent, and can be modulated by the precise composition of the protein complex to which they are recruited. Therefore, the degree of cross-talk implied by the results presented here between the signalling...
pathways of SAGM supplements during type II cell differentiation is not surprising.

However, our results suggest that type II cell differentiation may be particularly improved by the removal of retinoic acid and/or T3 from the differentiation medium. Retinoid signalling has been shown to exhibit stage-specific effects on mouse lung development, being essential for septation and formation of the primary lung buds in the early embryo, and stabilizing bronchial tubules and inhibiting the formation of distal lung structures at the pseudoglandular stage (Beers et al., 1998; Chazaud et al., 2003; Mollard et al., 2000). Retinoid metabolites have also been shown to inhibit directly the expression of the murine surfactant protein C gene (George and Snyder, 1997; Metzler and Snyder, 1993). The finding that exclusion of retinoic acid from ES cell differentiation medium increased the expression of the alveolar type II cell specific gene SPC suggests that retinoic acid may promote the maturation of proximal lung lineages at the expense of distal phenotypes in the final stages of ES cell differentiation. It would be interesting to discover whether the expression of proximal lung-specific markers inversely correlates with SPC expression in response to retinoic acid supplementation of SAGM once suitably phenotype-specific bronchial epithelial markers have been identified. Less evidence is available for the regulatory effect of T3 on lung development, with a single study showing that T3 inhibits branching morphogenesis in explant mouse embryonic lung cultures, thereby prematurely accelerating the restriction of SPC expression to the few distal airways that have formed (Archavachotikul et al., 2002). However, transgenic mouse models and clinical cases of thyroid hormone signalling disorders have demonstrated that these hormones have a wide-ranging influence over development (Brent, 2000; O’Shea and Williams, 2002). Therefore, the effects of T3 on the differentiation of mES cells to lung phenotypes are likely to be secondary to those promoting the formation of other cell lineages, thus decreasing the relative size of the SPC-expressing population in the presence of T3.

A large degree of variability in SPC RNA expression was observed in this study between replicates of cell cultures which had undergone precisely the same differentiation protocol. This made data analysis difficult as most changes in SPC expression were rendered statistically insignificant by the wide standard deviations between replicates. It does not, however, follow that there were no genuine changes in gene expression; statistical significance only provides reassurance that this is highly unlikely. Considering the wealth of literature which would support a negative effect of retinoic acid and T3 on type II cell differentiation, we conclude that it is probable that the observed increase in SPC expression was indeed representative of a genuine increase in SPC gene transcription.

The inter-replicate variability we observed in this study may have been due to a variety of factors. Firstly, the differentiation method employed here may be a source of variation due to the lengthy period of spontaneous differentiation during which the ES cells in each sample may simply have formed different proportions of pneumocyte-progenitor cells. To compound this problem, long-term spontaneous differentiation of EBs in adherent culture is also associated with tight clustering of the differentiated progeny, which then become prone to spontaneous detachment from the culture substratum but are also resistant to trypsin dispersion. The growth factors and hormones present in the basic serum-containing medium inevitably cause uncontrolled differentiation, yet differentiating ES cells require serum for survival and we have observed that SAGM cannot be introduced earlier in the culture protocol. Developing a differentiation protocol which yields more uniform and controlled results is likely to prove challenging, however work is in progress to shorten culture time in particular. An alternative explanation for inter-replicate variation is that, as differentiation is a highly dynamic process, replicate cell cultures had achieved different degrees of differentiation at the point of harvesting. Most methods of assessing cellular differentiation entail the destruction of the culture, preventing a continual monitoring of differentiation state. However, our recent results suggest that Raman spectroscopy (Notingher et al., 2002, 2003), a technique which quantifies the DNA, RNA and protein content of living cells, may have a use in assessing the differentiation state of ES cell cultures, and this technology is currently under evaluation.

This is the first study to attempt to detect quantitatively the formation of a definitive endodermal cell lineage from ES cells by real-time RT-PCR. Previous works demonstrating the differentiation of ES cells into endodermal lineages such as liver and pancreas have established the
presence of that cell type through immunocytochemistry or non-quantitative RT-PCR (Lumelsky et al., 2001; Reubinoff et al., 2001; Ruhnke et al., 2003). Those that have succeeded in quantifying transcription factors tend to deal with mesodermal cell types that are formed with high efficiency by ES cells, such as osteoblasts and cardiomyocytes (Fijnvandraat et al., 2003; Wobus et al., 1997; zur Nieden et al., 2003). This work indicates that the type II cell phenotype is suppressed in the presence of T3 or retinoic acid and presents an initial step towards the identification of a defined medium for the differentiation of ES cells into alveolar epithelium for the regenerative treatment of lung disease.

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