Optimization of a serum-free culture medium for mouse embryonic stem cells using design of experiments (DoE) methodology

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Abstract The in vitro culture behaviour of embryonic stem cells (ESC) is strongly influenced by the culture conditions. Current culture media for expansion of ESC contain some undefined substances. Considering potential clinical translation work with such cells, the use of defined media is desirable. We have used Design of Experiments (DoE) methods to investigate the composition of a serum-free chemically defined culture medium for expansion of mouse embryonic stem cells (mESC). Factor screening analysis according to Plackett–Burman revealed that insulin and leukaemia inhibitory factor (LIF) had a significant positive influence on the proliferation activity of the cells, while zinc and L-cysteine reduced the cell growth. Further analysis using minimum run resolution IV (MinRes IV) design indicates that following factor adjustment LIF becomes the main factor for the

survival and proliferation of mESC. In conclusion, DoE screening assays are applicable to develop and to refine culture media for stem cells and could also be employed to optimize culture media for human embryonic stem cells (hESC).

Keywords Design of experiments · Mouse embryonic stem cells · Expansion · Culture medium optimization · Growth factors

Abbreviations

BMP4	Bone morphogenic protein 4
CDM	Chemically defined medium
DoE	Design of experiments
ESC	Embryonic stem cells
hESC	Human embryonic stem cells
LIF	Leukaemia inhibitory factor
MEF	Mouse embryonic fibroblast
mESC	Mouse embryonic stem cells
MinRes IV	Minimum run resolution IV
PTP	Protein-tyrosine phosphatases
ROS	Reactive oxygen species
SSC	Sum of squares

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Introduction

Embryonic stem cells (ESC) are derived from the inner cell mass of mammalian embryos in the early blastocyst stage. In vitro culture was described for

mouse embryonic stem cells (mESC) more than 20 years ago (Evans and Kaufman 1981; Martin 1981), and for human embryonic stem cells (hESC) in 1998 (Thomson et al.). ESC have the ability to grow indefinitely while maintaining pluripotency and they are capable to differentiate into cells of all three germ layers, i.e. ectoderm, mesoderm and endoderm (Bradley et al. 1984). These characteristics make ESC a potential source of differentiated cell types for therapeutic application in regenerative medicine. To enable the potential clinical use of ESC, experimental methods to generate sufficient cell amounts (Gerlach et al. 2010a) for subsequent differentiation into the desired cell type (Gerlach et al. 2010b) are needed, considering potential clinical safety aspects of produced cell preparations. Thus, the use of culture media avoiding serum and further animal-derived components, e.g. animal feeder cells, is a challenge.

Most culture models for ESC are still based on the use of serum, feeder cells or other animal-derived medium additives. However, it has been shown that it is possible to derive and propagate mESC in the absence of feeder cells when leukemia inhibitory factor (LIF) is added to the medium (Nichols et al. 1990). More recently successful maintenance of mESC in a serum-free culture medium was reported (Nichols and Ying 2006). Besides LIF, other growth factors like bone morphogenetic protein 4 (BMP4) were suggested to prevent mESC differentiation while avoiding serum addition (Ying et al. 2003). Johansson and Wiles (1995) proposed a chemically defined medium (CDM) composition, in which serum components required for cell growth are replaced by specific combinations of hormones, nutrients and purified serum proteins; and mESC survive in this serum-free medium for at least 6 days. The basic CDM consists of Iscove's modified Dulbecco's Medium plus Ham's F12 medium at a 1:1 ratio with addition of lipids, transferrin, insulin and LIF. By including polyvinyl alcohol the use of proteins could be avoided and replacement of bovine serum albumin (BSA) was possible (Wiles and Johansson 1999).

For further improvement of culture media for mESC proliferation, investigations on concentrations of individual medium components are of interest.

Current approaches for medium optimization are generally based on successively changing one or more factors in order to assess their effect on the response variables. The most common method is

based on one-factor-at-a-time experiments, which vary only one factor or variable at a time while keeping the others fixed (Xu et al. 2003). Thereby medium additives are successively adjusted and fixed in their optimum levels. These methods, however, require a large number of runs for achieving a high precision in effect estimation, and possible interactions cannot be determined (Frey et al. 2003). Thus, optimum levels of individually tested factors have to be frequently revised and adjusted during the optimization process.

To address this limitation, the methodology of statistical design of experiments (DoE) was originally developed by Fisher (1926). DoE analysis is based on the variation of multiple components in the same experiment according to a design matrix, which allows identification of factor interactions and thereby enabling more efficient adjustment of culture medium additives. Although DoE methods have been used since the mid-20th century, their application in the discipline of medium optimization has only recently been introduced. Thus far, the main area of application of DoE methods was the optimization culture media for cell growth and protein production, needed for example for the production of antibiotics (Mandenius and Brundin 2008). More recently, DoE methods have also been successfully used to study the effects of cell culture parameters of biotechnologically important cell types including different types of stem cells (Lee et al. 1999; Dong et al. 2008; Prudhomme et al. 2004; Chang and Zandstra 2004).

This study was directed to advance a serum-free defined culture medium for ESC, using DoE methods for screening of different medium factors. In addition, the suitability of the different medium variants under investigation for feeder-free culture was tested. Mouse ESC (mESC) were used as a model system for demonstrating the practicability of the method for medium optimization. Emphasis was placed on soluble cytokines and low-molecular medium components with a possible impact on mESC self-renewal. Eleven factors were investigated, including L-cysteine, C1-metabolites, transferrin, LIF, insulin, calcium (Ca^{2+}), zinc (Zn^{2+}), defined lipids, BMP4, transferrin supplement and L-carnosine. The approach was based on a two-step strategy, using the Plackett–Burman design for selecting the most effective factors out of the eleven factors under investigation,

followed by a minimum run resolution IV (MinRes IV) design for assessment of possible interactions between the selected factors. The effect of different media on the cell proliferation rate used as response variable was quantified by photometric determination of their metabolic activity. In addition, the effect of medium variation on cell morphology and expression of pluripotency markers was investigated.

Materials and experimental methods

Cells and culture procedures

During the experiments two mouse embryonic stem cell (mESC) lines, ESD3 (LGC Promochem, Teddington, UK) and 129/SVEV (Millipore, Billerica, MA, USA) were used. The ESD3 cell line was cultured at 37 °C and 5% CO₂ in 25 cm² cell culture flasks (TPP, Trasadingen, Switzerland) coated with 0.1% gelatine (Biochrom, Berlin, Germany) without feeder cells. 129/SVEV mESC were seeded at a cell density of 3.5×10^4 cells/cm² into 175 cm² cell culture flasks (BD Biosciences, San Jose, CA, USA) coated with 0.1% gelatine (Millipore). The cells were co-cultured with inactivated mouse embryonic fibroblasts (MEF) CD-1 (passage 5, density of 3.0×10^4 cells/cm²) isolated according to protocols from the WiCell Research Institute (Madison, WI, USA).

Cultures were maintained in DMEM containing 1,000 U/mL LIF (ESGRO), 15% fetal calf serum (FCS), 2 mmol/L L-glutamine, 71.4 µM beta-mercaptoethanol, non-essential amino acids (NEAA), nucleosides, and 100 U/mL antibiotics (penicillin/streptomycin). LIF (ESGRO), FCS, NEAA, and beta-mercaptoethanol were purchased from Millipore, while all other medium components were provided by Biochrom. For routine passaging, cells were detached enzymatically by incubation with 0.05%/0.02% (w/v) Trypsin/EDTA (Biochrom).

Medium factors investigated

The experimental media were based on the medium composition proposed by Johansson and Wiles (1995). The following medium factors were screened: L-cysteine HCl H₂O (Ajinomoto foods Europe SAS Hamburg Branch, Hamburg, Germany),

C1-metabolites (L-methionine [Ajinomoto foods], choline chloride [Sigma–Aldrich, St. Louis, MO, USA], folic acid [Hoffmann-La Roche AG, Basel, Switzerland], vitamin B₁₂ [Merck, Darmstadt, Germany], pyridoxal phosphate [Sigma–Aldrich]), LIF and human recombinant insulin (both from Millipore), CaCl₂·2H₂O and ZnSO₄·7H₂O (both from Merck), defined lipids (Invitrogen, Karlsruhe, Germany), partially Fe³⁺-saturated human transferrin and L-carnosine (both from Sigma–Aldrich), transferrin supplement Proxylferrin (Biochrom) and BMP4 (ProSpec-Tany TechnoGene, Rehovot, Israel). These factors were added to the medium in two levels (high or low) according to the experimental design (see Table 1).

Experimental design and statistical evaluation

A two-level fractional factorial design according to Plackett–Burman (resolution III design) was set up for eleven different factors in the first screening. Therefore $N = 12$ experimental medium compositions were used ($N - 1$ variables in N runs, where N is a multiple of 4). The layout for this two-level design is shown in Table 2, using the standard notation +1 and −1 to denote the “high” and the “low” level for each factor.

Statistical analyses were performed using GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA, USA) or the software package SPSS (SPSS Inc., Chicago, Illinois, USA). Values of MTT analysis were calculated as means ± standard deviations. The unpaired t-test was performed to compare the responses of experimental media with that of the reference medium no. 7. An univariate Analysis of Variance (ANOVA) was performed to identify significant differences among the different groups. Results from statistical analysis were used to rank the factors investigated with respect to their effect on cell proliferation.

On the basis of the results, seven factors were selected and further analysed with respect to possible factor interactions using MinRes IV design (resolution IV). According to the design principle, $N = 14$ experimental media (N variables in $2N$ runs) were tested, which varied in the concentrations of the seven factors under investigation (see Table 3).

Design and analysis of the MinRes IV design were performed using the software package Design Expert

Table 1 Concentrations of variable factors in the media designed according to Plackett–Burman or to MinRes IV design in comparison to the medium composition published by Johansson and Wiles (1995)

Factor no.	Design Variable factors	Plackett–Burman		Johansson and Wiles	MinRes IV	
		High	Low	CDM-Pattern	High	Low
1	L-carnosine ^a	7.5 mM	0	Low	0	0
2	L-cysteine ^b	0.5 mM	0.1 mM	Low	0.5 mM	0.1 mM
3	C1 components ^{b,c}	5 mL/L	2 mL/L	Low	NV	NV
4	Human transferrin ^b	15 mg/L	0	High	25 mg/L	15 mg/L
5	Transferrin supplement ^{a,d}	1 mL/L	0	Low	0	0
6	LIF ^b	1,000 U/mL	1 U/mL	Low	1,000 U/mL	1 U/mL
7	Insulin ^b	7 mg/L	1 mg/L	High	14 mg/L	7 mg/L
8	BMP4 ^a	10 μ L	0	Low	10 μ g/L	0
9	CaCl ₂ (calcium) ^b	0.9 mM	0.3 mM	High	1.5 mM	0.9 mM
10	ZnSO ₄ (zinc) ^b	1.5 μ M	0.5 μ M	High	NV	NV
11	Lipids ^{b,e}	10 mL/L	1 mL/L	High	15 mL/L	10 mL/L

NV not varied, factors included in CDM-Pattern

^a Newly introduced factors

^b Factors from medium composition by Johansson and Wiles

^c L-methionine, choline, folic acid, vitamin B₁₂, pyridoxal phosphate

^d ferric citrate, citric acid, sodium citrate, zinc sulfate, sodium EDTA

^e defined lipids (Invitrogen)

Table 2 Plackett–Burman design for 11 factors (12 testing media)

Media no.	L-carn.	L-cyst.	C1-comp.	Human transf.	Transf. suppl.	LIF	Insulin	BMP4	Calcium	Zinc	Lipids
1	<i>+I</i>	<i>+I</i>	<i>+I</i>	<i>+I</i>	<i>+I</i>	<i>+I</i>	<i>+I</i>	<i>+I</i>	<i>+I</i>	<i>+I</i>	<i>+I</i>
2	−1	<i>+I</i>	−1	<i>+I</i>	<i>+I</i>	<i>+I</i>	−1	−1	−1	<i>+I</i>	−1
3	−1	−1	<i>+I</i>	−1	<i>+I</i>	<i>+I</i>	<i>+I</i>	−1	−1	−1	<i>+I</i>
4	<i>+I</i>	−1	−1	<i>+I</i>	−1	<i>+I</i>	<i>+I</i>	<i>+I</i>	−1	−1	−1
5	−1	<i>+I</i>	−1	−1	<i>+I</i>	−1	<i>+I</i>	<i>+I</i>	<i>+I</i>	−1	−1
6	−1	−1	<i>+I</i>	−1	−1	<i>+I</i>	−1	<i>+I</i>	<i>+I</i>	<i>+I</i>	−1
7	−1	−1	−1	<i>+I</i>	−1	−1	<i>+I</i>	−1	<i>+I</i>	<i>+I</i>	<i>+I</i>
8	<i>+I</i>	−1	−1	−1	<i>+I</i>	−1	−1	<i>+I</i>	−1	<i>+I</i>	<i>+I</i>
9	<i>+I</i>	<i>+I</i>	−1	−1	−1	<i>+I</i>	−1	−1	<i>+I</i>	−1	<i>+I</i>
10	<i>+I</i>	<i>+I</i>	<i>+I</i>	−1	−1	−1	<i>+I</i>	−1	−1	<i>+I</i>	−1
11	−1	<i>+I</i>	<i>+I</i>	<i>+I</i>	−1	−1	−1	<i>+I</i>	−1	−1	<i>+I</i>
12	<i>+I</i>	−1	<i>+I</i>	<i>+I</i>	<i>+I</i>	−1	−1	−1	<i>+I</i>	−1	−1

+I and −1 denote the “high level” (marked in *italics*) and the “low level” (marked in **bold**) of a factor concentration

7.0 (STATCON, Witzenhausen, Germany), which is a program tailored for mathematic modelling and design. Based on ANOVA testing for analysing the experimental data, factors ranking lists were generated with the program including graphical illustration of statistical data, and possible factor interactions were determined.

Experimental testing of culture medium variants

After reaching confluence, cells were trypsinized using soybean inhibitor (Sigma–Aldrich) instead of FCS after enzymatic dissociation to avoid serum traces in the used cell suspension. Cells were seeded as described above into 96-well plates (TPP) or

Table 3 MinRes IV design for seven factors (14 testing media)

Media no.	L-cysteine	Human transf.	LIF	Insulin	BMP4	Calcium	Lipids
1	−1	−1	−1	<i>+1</i>	<i>+1</i>	−1	<i>+1</i>
2	−1	−1	<i>+1</i>	<i>+1</i>	−1	<i>+1</i>	−1
3	−1	<i>+1</i>	−1	<i>+1</i>	<i>+1</i>	−1	−1
4	<i>+1</i>	−1	<i>+1</i>	−1	<i>+1</i>	−1	−1
5	−1	<i>+1</i>	<i>+1</i>	−1	<i>+1</i>	<i>+1</i>	<i>+1</i>
6	−1	<i>+1</i>	−1	<i>+1</i>	−1	<i>+1</i>	<i>+1</i>
7	<i>+1</i>	−1	−1	−1	<i>+1</i>	<i>+1</i>	−1
8	<i>+1</i>	<i>+1</i>	<i>+1</i>	−1	−1	<i>+1</i>	−1
9	<i>+1</i>	<i>+1</i>	−1	−1	<i>+1</i>	−1	<i>+1</i>
10	<i>+1</i>	−1	<i>+1</i>	−1	−1	<i>+1</i>	<i>+1</i>
11	<i>+1</i>	<i>+1</i>	<i>+1</i>	<i>+1</i>	<i>+1</i>	<i>+1</i>	<i>+1</i>
12	<i>+1</i>	−1	−1	<i>+1</i>	−1	−1	−1
13	−1	<i>+1</i>	<i>+1</i>	<i>+1</i>	−1	−1	<i>+1</i>
14	−1	−1	−1	−1	−1	−1	−1

+1 and *−1* denote the “high level” (marked in italics) and the “low level” (marked in bold) of a factor concentration

24-well plates (BD Biosciences) for the screening studies. Lumox multiwell plates (Greiner Bio-One, Solingen-Wald, Germany) were used for immunofluorescence studies. Experiments were started by adding the different media to the wells according to the design matrix (Tables 2 and 3). Tests for evaluation of media effects were performed once the control cells maintained in the standard culture medium reached confluence (day 5, or culture day 3 and 6 when cells were passaged). In the screening part all experiments were carried out in duplicates using different wells, and repeated twice on different plates. The experimental order was randomized to minimize unpredictable errors.

Parameters for evaluation of medium effects

Light microscopy

Microscopic evaluation of the cells was carried out regularly during the whole experimental period using a phase contrast light microscope (Axiovert 135, Carl Zeiss, Göttingen, Germany) and a Coolpix 4500 camera (Nikon GmbH, Düsseldorf, Germany). Cultures were characterized by cell density, shape, size, cytoplasm-to-nucleus ratio and cytoplasmic granulation.

MTT assay

Cell proliferation rates were determined by means of the MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. MTT analyses were

performed using ready-to-use test kits (Sigma–Aldrich) for the 24-well plates or according to the modified protocols for the 96-well plates. In the modified approach, cells were treated with MTT solution (AppliChem, Darmstadt, Germany) for 3 h followed by blocking with isopropanol-hydrochloric acid solution (Merck). The absorbance of each well was measured at a wavelength of 570 nm using an automatic microtiter plate reader (MRX-TC Revelation, Dynex Technologies, Chantilly, VA, USA). The measured optical density was used as response variable for the statistical evaluation.

Immunofluorescence

Staining procedure: The cultured cells were fixed and stained directly in the lumox multiwell plates. Cells were fixed with 4% formaldehyde solution (Herbeta-Arzneimittel, Berlin, Germany) for 10 min at room temperature, washed once with phosphate buffered saline (PBS) and permeabilized with 80% methanol (Mallinckrodt Baker, Phillipsburg, NJ, USA) for 20 min at -20°C . All following steps were carried out at room temperature. After rinsing with PBS, cells were incubated for 60 min in blocking buffer consisting of PBS supplemented with 2% FCS, 3% bovine serum albumin (BSA, Sigma–Aldrich) and 0.2% Teleostean gelatin (Sigma–Aldrich). Primary antibodies diluted in blocking buffer were added and incubated for 60 min. Subsequent to threefold washing with PBS, the secondary antibodies diluted

in blocking buffer were added to the cells and incubated at room temperature for another 60 min. Afterwards the wells were washed three times and incubated for 5–10 min with 4',6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich) diluted 1:5,000 in PBS. Wells were mounted with Aqua Polymount solution (Polysciences Inc., Washington, PA, USA). An inverse microscope (Axiovert 200 M, Carl Zeiss) equipped with a digital camera (MicroPublisher 3.3, Weiss Imaging, Bergkirchen, Germany) was used for microscopic observation. Pictures were acquired and processed using the digital imaging software Image Pro Plus (Weiss Imaging).

Antibodies and dilutions: Primary antibodies: mouse anti-mouse Oct 3/4 (1:200), Santa Cruz Biotechnology, Santa Cruz, CA, USA; mouse anti-mouse SSEA-1 (1:20), Developmental Studies Hybridoma Bank, University of Iowa, IA, USA. Secondary antibodies: goat anti-mouse IgG Cy2 (1:1,000); goat anti-rabbit IgG Cy3 (1:1,000), both from Dianova, Hamburg, Germany.

Flow cytometry

Staining procedure: For flow cytometry analysis 10^7 viable cells were suspended in 10 mL of cold 4% formaldehyde and incubated on ice for 10 min. After washing the cells 1 mL of 10% normal goat serum blocking solution (NGS, Invitrogen) was added to each tube and cells were incubated on ice for at least 30 min. 2×10^6 cells were used for each antibody staining. Prior to staining with the intracellular marker Oct 3/4 cells were permeabilized with a 0.1% saponin solution (Sigma–Aldrich) for 15 min. The cells were incubated sequentially for 30 min on ice with the primary antibodies and fluorochrome coupled secondary antibodies, both diluted in 10% NGS. Between each incubation step, cells were washed three times using 5% NGS. The stained cells were dissolved in 500 μ L of PBS for measurement. Flow cytometry data were acquired on a FACScalibur device (BD, Franklin Lakes, NJ, USA) and analyzed by Cell Quest Pro software (BD Biosciences).

Antibodies and dilutions: Phycoerythrin (PE) conjugated anti-SSEA-1 mouse IgM (R&D systems, Minneapolis, MN, USA), mouse anti-Oct 3/4 IgG (BD) and Alexa Fluor 488 (Invitrogen).

Results

Plackett–Burman design: mESC cultured with or without MEF

In the first step of culture medium optimisation using the DoE methodology eleven factors were investigated for selecting the most important factors for mESC proliferation. Factors were varied using a Plackett–Burman design based on a published media formulation (Johansson and Wiles 1995). Concentrations of the above factors were set as shown in Table 1. Experiments were performed in mESC cultures maintained without or with feeder cells. The MTT assay was used as a quantitative response variable. In addition, the cell morphology and the expression of pluripotency markers were investigated.

MTT assays were performed on day 5 in mESC monocultures or on day 3 (passage) and day 6 in the feeder cell cocultures. The results of MTT testing are shown in Fig. 1. Means of values were normalized with respect to the published medium (no. 7). In both culture models, the media no. 1 and no. 3 showed higher MTT values than medium no. 7. Furthermore the feeder-free cultures incubated with medium no. 4 showed almost three times higher values in comparison with the published medium formulation (no. 7). All three media were characterized by high levels of insulin and LIF, while they differed in the content of the other factors (Table 2). Medium no. 5 showed a similar MTT response than the reference medium, while the other media showed a lower response. The poorest outcome, especially in feeder-free cultures, was observed in the group treated with medium no. 2, which is characterized by high L-cysteine and zinc concentrations and low levels of BMP4, lipids and calcium (Table 2). Correlation analysis showed that the responses of mESC (129 SVEV) cultured with MEF and passaging and those of mESC (ESD3) cultured without MEF and without passaging are significantly correlated ($R^2 = 0.872$, $p < 0.001$). However, both positive and negative effects of the experimental media on the cell proliferation activity were more pronounced in feeder-free mESC cultures than in cultures maintained with MEF.

The results from microscopic observation confirmed these findings (Fig. 2). Cultures maintained without feeder cells in medium no. 1, 3 or 4 showed a higher cell density, a better demarkation of

Fig. 1 MTT responses (optical density) of mESC cultured without or with feeder cells (MEF) in 12 different medium compositions using the Plackett–Burman design. Values (means $\pm$ SD) were normalized to the reference medium no. 7. The unpaired *t*-test was performed to compare each group with the reference medium no. 7

Fig. 2 Light microscopic pictures of mESC cultures maintained in different experimental media. **a, b** mESC cultured without MEF over 5 days in medium no. 4 (**a**) or medium no.

12 (**b**); magnification 50 $\times$. **c, d**: mESC co-cultured with MEF over 6 days in medium no. 1 (**c**) or reference medium no. 7 (**d**); magnification 50 $\times$

colonies and a lower proportion of cell debris compared with the other cultures, as shown representatively for medium no. 4 (**A**) and no. 12 (**B**). A less pronounced effect of the medium composition on the culture morphology was observed in feeder-containing cultures, in accordance to the results from

metabolic assays. However, cultures with higher metabolic activities showed a tendency towards a higher cell density compared to those with a lower metabolic performance, as shown exemplarily for medium no. 1 (**C**) and the reference medium no. 7 (**D**).

Fig. 3 Immunofluorescence staining of the pluripotency marker Oct 3/4 (*red*). Nuclei are stained with DAPI (*blue*). Cultures of mESC were co-cultured with MEF (magnification 400 $\times$) or maintained without the addition of MEF (magnification

200 $\times$), using the reference medium (no. 7) or the experimental media no. 3/12 composed according to the Plackett–Burman design. Immunofluorescence studies were performed on the third day of culture

Immunofluorescence staining was performed to determine the proportion of undifferentiated cells in the cultures after incubation with the experimental media. Figure 3 shows immunofluorescence pictures of cultures maintained in the reference medium (no. 7), in medium no. 3 representative for media showing a positive effect as determined by MTT testing, or medium no. 12 representative for those with a negative effect on cell proliferation. The results revealed that most cells expressed the pluripotency marker Oct 3/4 while lacking or just slightly expressing SSEA-1 (not shown). There were no

distinct differences in marker expression between the different experimental media.

Based on MTT assay results, ANOVA was performed and the sum of squares (SSC) were calculated. Based on the SSC values, ranking lists of the eleven factors investigated were generated for feeder-free and feeder-containing mESC cultures, respectively (Table 4). In cultures maintained without feeder cells, insulin, LIF, zinc, L-cysteine, human transferrin, BMP4 and L-carnosine (in decreasing order) had a significant impact on the response ($p < 0.05$). The effect was positive for insulin, LIF,

Table 4 Ranking list of the factors investigated with respect to their effect on mESC cultured without or with feeder cells (MEF) using the Plackett–Burman design

mESC cultured without MEF day 5				mESC co-cultured with MEF means of days 3 and 6			
Factor	SSC	Effect	Significance	Factor	SSC	Effect	Significance
Insulin	5.749	+	***	Insulin	1.168	+	***
LIF	3.440	+	***	Calcium	1.051	+	***
Zinc	3.317	–	***	BMP4	0.833	+	***
L-cysteine	2.929	–	***	L-cysteine	0.711	–	*
Human transferrin	2.521	+	***	LIF	0.583	+	**
BMP4	2.269	+	***	Lipids	0.424	+	*
L-carnosine	0.985	+	*	Zinc	0.418	–	/
C1-components	0.591	–	/	C1-components	0.398	+	/
Lipids	0.414	+	/	Transferrin supplement	0.359	+	/
Transferrin supplement	0.203	+	/	Human transferrin	0.349	+	/
Calcium	0.144	+	/	L-carnosine	0.123	–	/

The table shows the sum of squares (SSC)-values of the different factors calculated by ANOVA. Positive or negative effects on the cell proliferation (MTT assay) are marked with + or –. Effects were judged as significant, when the p-value was lower than 0.05

Values of Significance: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

human transferrin, BMP4 and L-carnosine, while zinc and L-cysteine had a negative effect on cell proliferation as determined by MTT testing. In mESC cocultured with MEF as feeder cells, insulin, calcium, BMP4, LIF and lipids had a significant positive influence, while L-cysteine had a negative effect. Thus, in both culture models insulin represents the main factor with a positive influence on the proliferation rate of mESC. The comparison of feeder-free and feeder-containing cultures showed that the effect of several factors depends on the presence of MEF. For example, in both culture models zinc had a negative impact on the response, but it was ranked in different positions. Calcium occupied the last ranking position in the cultures without MEF indicating a low influence, but showed a significant positive effect in cultures with MEF (ranking position 2). Other factors, like insulin, L-cysteine, C1 components or transferrin supplements showed a comparable influence in both culture models.

MinRes IV design: mESC cultured without MEF

On the basis of the results from the Plackett–Burman design, seven of the eleven factors investigated were selected for further medium optimization studies using the MinRes IV approach (Table 1). Concentrations of the two levels (“high” and “low”)

investigated were further increased for calcium, lipids, insulin and transferrin. Levels of LIF and BMP4 were not changed since the concentrations used were already at the maximal level recommended for mESC cultivation. Concentrations of zinc, C1 components and L-carnosine were in accordance with those suggested by Johansson and Wiles. Despite its negative effect L-cysteine was again varied in the previously used concentration levels because of its assumed interaction with insulin. In contrast, transferrin supplement were omitted due to no effect (Fig. 4).

Again a two-level fractional factorial design was set up for the seven different factors, leading to 14 different media compositions (Table 3). Experiments were only performed with feeder-free mESC cultures (culture period = 5 days), because the results from the first step of medium optimization had shown that the effects of influencing factors were more obvious in that culture model.

In Fig. 5 the responses of the MTT assay performed in mESC cultures incubated with the 14 experimental media are graphically illustrated. The media no. 2, 4, 5, 8, 10, 11, and 13 led to a markedly improved growth of the mESC with at least by 50% higher responses than the medium composed according to the original formulation, which is represented by the medium no. 14. All these media were

Fig. 4 Application of the two-step strategy using the Plackett–Burman design in the first screening followed by factor selection and further adjustment using the MinRes IV design

Fig. 5 MTT responses (optical density) of mESC cultured without or with feeder cells (*MEF*) in 14 different media composed using the MinRes IV design. Values (means $\pm$ SD) were normalized to the reference medium no. 14. The unpaired *t*-test was performed to compare each group with the reference medium no. 14

characterized by high levels of LIF (1,000 U/mL), while they differed in the content of the other factors. The comparison of independent runs showed a high inter-test reliability ($r_s = 0.771$).

The findings from microscopic inspection corresponded well to the results of the MTT assay. Cells cultivated in media with a high level of LIF were more confluent than cells cultivated with a low level of LIF (not shown). Immunofluorescence analysis of

pluripotency markers showed expression of Oct 3/4 in most cells, while immunoreactivity of SSEA-1 was only scarcely present (not shown).

For additional characterisation of the marker expression profile of the cells, flow cytometry analysis of cultured cells was performed. Figure 6 exemplarily shows the density dot plots and the arrangement of fluorescence signals after cell labelling with antibodies against Oct 3/4 or SSEA-1 for

Fig. 6 Flow cytometry analysis including scatter plot (red) and staining of the pluripotency markers Oct 3/4 (blue) or SSEA-1 (pink) in mESC cultured in medium no. 13 or medium no. 14. Studies were performed on the third day of culture

the media no. 13 (high level of LIF) and no. 14 (low level of LIF). 94.66% of the gated cells were positive for Oct 3/4 and 50.84% for SSEA-1, when mESC were cultured in media no. 13. When mESC were cultured in media no. 14, they showed a positive expression profile for Oct 3/4 in 98.13%, but only 28.95% of the mESC were positive for SSEA-1. Thus, FACS analysis confirmed the findings of the proliferation assay.

For factor analysis the data of calculated MTT responses were entered into the Design Expert software. In Table 5 the mean values of all seven factors are ranked from the highest to the lowest effect. The SSC values clearly show that only LIF had a significantly (positive) influence with a contribution of 86.94%, while the other factors showed no significant positive or negative effect, with contributions in the range of 0.27 to 2.96%. In the half-normal plot shown in Fig. 7 each square depicts the effect by individual factors or two-factor interactions upon the

Table 5 Ranking list of the factors investigated with respect to their effect on mESC cultured without feeder cells (MEF) using the MinRes IV design

mESC cultured without MEF				
Means of day 5				
Factor	SSC	Effect	Contribution (%)	Significance
LIF	0.0935	+	86.94	***
L-cysteine	0.0037	+	2.96	/
Lipids	0.0025	–	2.37	/
Calcium	0.0015	+	1.33	/
BMP4	0.0012	+	1.21	/
Insulin	0.0006	–	0.62	/
Human Transferrin	0.0003	–	0.27	/

The table shows the sum of squares (SSC)-values of the different factors calculated by ANOVA. Positive or negative effects on the cell proliferation (MTT assay) are marked with + or –. Effects were judged as significant, when the p-value was lower than 0.05

Values of Significance: *** $p < 0.001$

Fig. 7 Half-normal plot of the group effects for run no. 1 in the MinRes IV design. Each square depicts the effect by one group or individual factor interaction. Negative effects are marked by black squares, positive effects by gray squares

final optical density. The graph illustrates the prominent effect of LIF on cell proliferation. The effects by other factors were within the random variance.

Discussion

In order to be successful in DoE screening it is vital to select suitable concentration ranges for the factors investigated to allow the detection of effects on the responses. Thus, the concentration values should cover a sufficiently broad range from a minimum to a maximum level where clear differences in factor effects are expected. For optimization of serum-free culture media for mESC in this study the initial concentrations of the components were based on literature data (Johansson and Wiles 1995). Using the means of MTT values a basic DoE evaluation of the effects of individual factors was carried out. The resulting data allowed a clear ranking of media component effects. In the Plackett–Burman design three of the eleven factors investigated, namely insulin, LIF and BMP4 had a significantly positive influence on the cell growth in both feeder-free and feeder-containing cultures. In addition, a positive effect by human transferrin and L-carnosine was observed in feeder-free cultures, while cultures maintained with MEF profited from calcium and lipid substitution. Based on these results, a MinRes IV design was used for further medium optimization and detection of possible factor interactions. Results from MinRes IV screening clearly showed the predominant role of LIF in mESC culture media, indicating that LIF becomes the most relevant factor for mESC proliferation, following factor adjustment especially with respect to insulin concentrations.

Insulin is a pancreatic hormone contained in many culture media for primary cells and cell lines due to its supportive effect on cell proliferation and differentiation *in vitro*. It was reported that insulin addition to culture media increases the cell number of cultivated mouse blastocysts (Mihalik et al. 2000) and of endogenous protein reserves in the murine embryo (Dunlison and Kaye 1993). The data from this study show that an increase of the insulin concentration from 1 to 7 mg/L has the most pronounced positive effect among the factors investigated on the proliferation of mESC. This result was independent of the cell line used (ESD3 or 129SVEV) or of MEF addition. Further increase of the insulin concentration to 14 mg/L in the second step using the MinRes IV design did not lead to further improvement of cell proliferation. Interestingly, at this insulin concentration, LIF was the most effective factor for mESC growth. Therefore, a

possible explanation for detecting no further effect of insulin in the MinRes IV design could be that it was masked by the influence of LIF.

LIF belongs to the family of interleukin 6 (IL-6)-type cytokines and is known to promote self-renewal and to inhibit differentiation (Smith et al. 1988). The influence of LIF on the proliferation of mESC was controversially discussed. Whereas Zandstra et al. (2000) reported that LIF supplementation had no effect on mESC growth *in vitro*, Viswanathan et al. (2003) from the same group reported that the addition of LIF resulted in a distinct dose-dependent survival and proliferation advantage. Regarding the results from Plackett–Burman design analysis performed in this study only mESC cultured in the media containing a “high level” of both LIF (1,000 U/mL) and insulin (7 mg/L) had a higher proliferation activity than the published medium composition in both culture models. Moreover, in the second step of analysis, only LIF had a significantly beneficial effect, indicating that following optimization of the other media components the impact of LIF becomes more prominent.

The third factor with an unequivocally positive effect on mESC proliferation was BMP4, which is well known as a potent epidermal inducer and neural inhibitor factor in vertebrate embryos (Wilson and Hemmati-Brivanlou 1995), but was also shown to stimulate cell proliferation *in vitro*, for example in mESC-derived endothelial cell cultures (Suzuki et al. 2008). During the first step of our screening analyses BMP4 showed a significantly positive influence on the proliferation behaviour of mESC, as determined by the MTT assay. However this effect was much higher in the feeder-containing than in feeder-free cultures. In the second step of analysis (MinRes IV design) the effect of BMP4 on cell proliferation was much lower, although still positive. In accordance with the results of this study, it was reported that the combination of LIF and BMP4 enhanced the self-renewal of ESC and enabled serum-free culture, resulting in highly pure populations of undifferentiated cells (Ying et al. 2003).

Besides Insulin, LIF and BMP4, both human transferrin and L-carnosine also exerted a significantly positive effect on mESC growth, although only in feeder-free cultures. Transferrin plays an important role in ferric ion delivery and also acts as an extracellular antioxidant. It is well known that transferrin supports the proliferation of cells cultivated under serum-free conditions and that it has a

positive influence on several cell lines (Chen et al. 1993).

Synthetic transferrin supplement was used as an alternative to natural human transferrin, with respect to clinical requirements. However, since transferrin supplement had no significant influence on mESC growth, in contrast to human-derived transferrin, it was omitted in the second step of analysis.

L-carnosine is a chelator of toxic metal ions as well as a potent antioxidant. Holliday and McFarland (2000) showed that L-carnosine promotes the growth of murine embryonic stem cells. Results from Plackett–Burman design analysis confirmed a positive influence of L-carnosine on cell proliferation in the feeder-free cultures. However, since a negative effect was observed in feeder-containing cultures, L-carnosine was not varied during MinRes IV analysis and used as suggested by Johansson and Wiles.

In contrast, both calcium and lipid substitution led to a significant proliferation advantage in the feeder-containing cultures investigated in the first step of analysis. Calcium acts as an intracellular messenger and is also known to facilitate the attachment of cells to substrates or to other cells by the modulation of the function of adhesion molecules, namely cadherins, selectins and integrins. Thus the observed positive effect of calcium in the first screening step could be due to improved adherence of mESC to the feeder layer and/or in the support of gene transcription processes. The positive influence of lipids can be attributed to the fact that they represent an important energy source for cells. Additional lipid supplementation is needed especially in serum-free culture media that are devoid of serum-derived lipids.

C1 components were selected based on theoretical considerations for the support of mESC in the serum free CDM, e.g. the C1 components are metabolites and cofactors for the methylation metabolism, which plays a major role in stem cell maintenance (Chen et al. 2003). However, since increased concentrations of C1 components showed no significant influence on the cell proliferation in both culture models, they were added at the original level suggested by Johansson and Wiles in the second step of medium optimization.

A negative influence on mESC proliferation was observed in the first step of analysis mainly when “high” concentrations of zinc or L-cysteine were used in the culture medium. This result was independent of

the cell line used (ESD3 and 129SVEV) and of the addition of MEF.

Zinc is essential for cell proliferation and differentiation, especially for the regulation of DNA synthesis and mitosis, and it is a structural constituent of a large number of proteins, including enzymes of cellular signalling pathways and transcription factors (Beyersmann and Haase 2004). The negative effect of zinc observed in this study could be explained by its possible interaction with insulin. In the presence of zinc within the cell, insulin monomers assemble to a dimeric form for storage as zinc crystal (Chausmer 1998). Thus, an increased zinc concentration could lead to enhanced formation of insulin-zinc crystals in association with a decrease in the concentration of free insulin as required for cell support. Due to its negative influence zinc was not varied in the medium composition in the second step of analysis.

In contrast, L-cysteine was further tested in the MinRes IV analysis despite its negative effect, since it plays an important role in protecting cells from oxidative stress and it is the limiting amino acid in the physiological synthesis of the antioxidant tripeptide glutathione. Interestingly, L-cysteine had no negative effect in the MinRes IV design, and even showed a tendency towards a positive influence on the proliferation of mESC in media with increased insulin concentrations. This could be explained with an interaction between insulin and L-cysteine as well, since the reduction of disulfide bonds through L-cysteine may inactivate the growth-promoting activity of insulin and high concentrations of insulin are necessary to achieve high cell numbers, as suggested by Bottenstein and Sato (1979).

L-cysteine, however, does not only indirectly interfere with the effect of insulin on cell growth but also in a direct manner via the reduction of intracellular reactive oxygen species (ROS). The redox state is a central modulator of the balance between self-renewal and differentiation in stem cells, as shown for glial precursor cells (Smith et al. 2000). ROS have important roles during cell signalling, for example they act as second messengers in the insulin signalling cascade (Goldstein et al. 2005). The NADPH oxidase NOX4 mediates the accumulation of cellular ROS and is thereby linked with effects of insulin on the oxidative inhibition of the protein-tyrosine phosphatases (PTP). As ROS react with the catalytic L-cysteine of PTP, they can also react with

free intracellular L-cysteine, which is influenced by the concentration of L-cysteine in the culture media. These changes in the redox state could be an explanation for the dominant role of LIF in the MinRes IV design and the changing ranking position of L-cysteine in the two designs.

To provide evidence that the mESC cultivated in this study were still pluripotent, the cells were characterized by microscopic observation and analysis of their antigenic properties. The cultures maintained in media with a positive effect on cell proliferation showed no significant changes in morphology, while the use of media with a negative effect on proliferation resulted in morphological alterations and decreased cell densities indicating cell damage.

Immunofluorescence analysis of the pluripotency markers Oct 3/4 and SSEA-1 in mESC cultures revealed immunoreactivity for Oct 3/4 in the majority of cells, while the immunofluorescence staining intensity for SSEA-1 was generally weak, and thus allowed no reliable evaluation of the pluripotent state of the cells. In contrast to the immunofluorescence staining for SSEA-1, quantitative flow cytometry analysis performed with cells from representative cultures showed a clear relation between the proportion of SSEA-1 positive cells and the proliferation rate measured. However, SSEA-1 expression was still low as compared with Oct 3/4 expression. The expression of SSEA-1 in mESC cultured in the presence of a “high level” (1,000 U/mL) of LIF was nearly twice as high (50.84%) than in mESC incubated with a “low level” (1 U/mL) of LIF (28.95%). In accordance to the results from immunofluorescence studies, the expression of Oct 3/4 was nearly similar with 98.13 to 94.66% in all samples.

Since SSEA-1 is a surface marker (Solter and Knowles 1978), while Oct 3/4 stains the nucleus, a possible explanation for the finding of low SSEA-1 expression as compared with Oct 3/4 is that this marker got partly lost during the cultivation and/or staining procedure. The cessation of SSEA-1 expression could also indicate starting differentiation, as shown by Smith et al. (1988). However, the results from Oct 3/4 staining confirmed the undifferentiated state of the cells, since this marker rapidly disappears in cultured embryos prior to any evidence of morphological differentiation (Buehr et al. 2003).

In conclusion, the DoE design methodology applied in this study allowed the identification and optimization

of critical medium components for mESC proliferation. The results show that insulin and, after initial adjustment of factor concentrations, LIF are the most effective compounds of the eleven factors investigated in supporting the proliferation activity of mESC in vitro. The employed two-step strategy facilitated gaining maximal information with only a minimum of experimental series. The application of the Plackett–Burman design enabled an initial screening of numerous possible influencing factors, minimizing the number of required runs. The combination of initial screening with following detailed analysis of selected factors by means of MinRes IV design provides the advantage that possible factor interactions, which are not detected by the Plackett–Burman design, can be elucidated (Kennedy and Krouse 1999). Since the DoE methodology has considerable advantages with respect to process yields, overall costs and development time compared with conventional approaches for medium optimization (Montgomery 2009), the use of this technique could largely enhance and improve the screening of media factors and further important culture parameters, like oxygen/carbon dioxide partial pressures, pH value or temperature conditions. This is of particular interest for hESC, which are extremely sensitive to their environmental conditions.

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