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Effect of chemopreventive agents on differentiation of mouse embryonic stem cells

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1. ABSTRACT

Chemopreventive agents are derived from edible plants and from ancient time is a part of daily intake for many humans and animals. There are several lines of compelling evidence from epidemiological, clinical and laboratory studies that these dietary constituents are associated in reducing cancer risks. However, developmental toxicity of these natural compounds cannot be excluded. In the present study, we examined the effect of chemopreventive agents on the differentiation of mouse embryonic stem cells (ESCs) as an *in vitro* embryotoxicity model. We assumed that inhibition of developmentally regulated genes *in vitro* might predict developmental toxicity also under *in vivo* conditions. We found that epigallocatechin gallate (EGCG) (20 μM) induced the expression of mesodermal and cardiomyocyte genes and a significant increase in the number and the percentage of cardiomyocytes. The increase of the subpopulation correlated with higher numbers of beating foci and beating frequencies. Curcumin on the other hand at 0.4 μM was seen to enhance expression of ectodermal transcripts. Quercetin (2.5 μM) was found to inhibit several developmentally regulated genes.

2. INTRODUCTION

Numerous publications report that both nutritive and nonnutritive plant-derived dietary factors can inhibit carcinogenesis in a mechanism called chemoprevention (1-4). A large proportion of the daily diet includes polyphenols that are found in many vegetables and also in the leaves, leaf buds and internodes of the tea plant (5). A large number of chemicals present in food plants are reported to interfere with specific stages of carcinogenesis and atherosclerotic development (3, 6-8). Multiple mechanisms have been shown to account for the beneficial actions of dietary constituents, but attention has recently been focused on signaling pathways targeted by chemopreventive phytochemicals (9, 10). In this context, it has been proposed that polyphenols exert their beneficial effects via their antioxidant activity against reactive oxygen species (ROS) (9, 10). Curcumin is a polyphenolic compound present in the rhizome of turmeric, a widely-used spice. It has a wide range of effects such as anti-inflammatory, antioxidant, antiproliferative and antiangiogenic effects (11, 12). Quercetin is a flavonoid and this compound class possesses significant antihepatotoxic, antiallergic, anti-inflammatory,

antiosteoporotic and antitumor activities (13-17). Beneficial health effects of quercetin, epigallocatechin gallates (EGCG) and related phenolic compounds found in tea, wines, and other plant products involves pharmacologic intervention to prevent, inhibit or reverse carcinogenesis or prevent the development of invasive cancer (18-20). Natural polyphenolic compounds reduce in general oxidative stress, which is the most likely mechanism in the protective effects of these compounds. Overall, chemopreventive agents have received much attention as dietary components with health benefits, but the effect on developmental toxicity are not yet studied.

Recently, embryonic stem cells (ESCs) have been recognized promising tools for basic research and regenerative medicine because of their ability to differentiate *in vitro* (21, 22) into the three germ layers (endoderm, mesoderm and ectoderm) and further to somatic cells such as neurons or muscle cells. There is increasing evidence that antioxidants influence the differentiation of ESCs and induced pluripotent stem cells (iPSCs) toward specific somatic cells. In this context it has been shown that the polyphenolic compound resveratrol induces osteogenesis in iPSCs (23) and hydrogen peroxide (H_2O_2) promoted the differentiation of ESCs to smooth muscle cells (24). In addition vitamin C synergistically with salvianolic acid B promoted cardiomyocyte development of ESCs (25). Additionally, ESCs offers one of the most promising *in-vitro* developmental toxicity models validated as the embryonic stem cell test (EST) (26-28) and has added advantages over the classical evaluation of chemical-induced developmental toxicity that require large number of laboratory animals (29). ESCs during *in vitro* differentiation recapitulate cellular developmental processes and gene expression patterns of early embryogenesis.

In the present study, we investigated in detail the effects of EGCG, curcumin and quercetin on the differentiation processes in murine ESCs. Here we show that EGCG promotes the differentiation of ESCs to mesodermal cell types including cardiomyocytes. Curcumin enhanced the differentiation of ESCs towards ectodermal cells.

3. MATERIAL AND METHODS

3.1. Embryonic stem cells lines and culture

Undifferentiated CGR8 cells (ECACC 95011018, ECACC, Salisbury, Wiltshire, UK) were propagated in Glasgow Minimum Essential Medium (GMEM, Life Technologies, Merelbeke, Belgium) supplemented with 0.05 mM beta-mecaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate and 10 % fetal calf serum (Life Technologies, Merelbeke, Belgium) as well as leukemia inhibitory factor (ESGRO-LIF, Millipore, Schwalbach, Germany) under feeder-free conditions (30) on gelatinized tissue culture flasks. CGR8 cells were dissociated by trypsinization and were passaged every second day to maintain confluence between 60 and 70 %. Cells were cultured in a humidified atmosphere of 5 % CO_2 at 37 °C.

3.2. Compounds

Curcumin, EGCG and quercetin were purchased from Sigma-Aldrich (Heidelberg, Germany) and were dissolved in dimethylsulfoxide (DMSO). The concentration of DMSO was kept less than 0.1% (v/v) in all experimental groups, and 0.1% DMSO was used as a vehicle control throughout the study. Additionally, 5-Flurouracil (5FU, Sigma-Aldrich, Heidelberg, Germany) was used as cytotoxic agent for cytotoxicity assessments.

3.3. Induction of ESCs differentiation

ESC differentiation was done (Figure 2A) as described previously (31). Briefly, the CGR8 cells were differentiated *in vitro* using the hanging-drop method to generate embryoid bodies (EBs) (32). Differentiation was induced by LIF withdrawal and by forming 20 µl hanging drops on 10 cm low adhesion dishes from trypsin-dissociated ESCs (2.5×10^4 cells / ml). Hanging drops were prepared in differentiation medium made of Iscove's modified Dulbecco's Medium (IMDM, Gibco, Eggenstein, Germany) supplemented with 20 % fetal calf serum, 1 % non-essential amino acids, 2 mM L-glutamine, and 100 µM beta-mercaptoethanol). Compounds or solvent control (DMSO) were added to the hanging drop medium. Plates were incubated at 37 °C and 5 % CO_2 in a humidified incubator for 2 days. EBs were washed from hanging drops and resuspended in differentiation medium and further incubated at 37 °C in 5 % CO_2 incubator under orbital shaking conditions (20 rpm), with a medium change every alternate day. The clusters increased in size during differentiation and appearance of spontaneously contracting cardiomyocyte clusters was monitored using an inverted phase contrast microscope (Zeiss Axiovert 25, Oberkochen, Germany). The beating clusters within the EBs were examined for 1) beating frequency measured manually by counting number of beats per minute in individual EBs and here defined as number of beats per minute 2) number of beating EBs. Videos of beating EBs were captured using a Sony camera (Sony- DFW-X700, Sony Corporation, Tokyo, Japan).

3.4. MTS toxicity assay

The growth inhibitory effect of curcumin, EGCG and quercetin on CGR8 cells was measured using 1) MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (33) and 2) Crystal violet staining assay (34). Cells were seeded at 1 x 103 cells in 96-well plates, after 24 h, the cells were treated with different concentration of curcumin, EGCG and quercetin (0.01 to 100 µM). After 72 h treatment, medium containing test compounds was removed, each well was washed twice with PBS and 200 µl of 2 mg/ml MTS (Promega, Heidelberg, Germany) containing phenazine methosulfate (PMS, Sigma-Aldrich, Heidelberg, Germany) was added into each well. The plates was incubated at 37 °C until purple color due to formazan dye was produced which was quantified in an ELISA micro plate reader (Tecan, Crailsheim, Germany) at an absorbance of 490 nm.

Crystal violet stains DNA, and the amount of dye taken up by cells is quantitated colorimetrically. For this assay, compound treated 96-well plates were washed twice

with PBS and 50 µl of 0.5 % crystal violet (Sigma-Aldrich, Heidelberg, Germany) dissolved in 20 % methanol (w/v) was added to each wells, and after 10 minute of incubation at RT each wells were washed thrice with PBS. The dye was eluted by incubating with 200 µl of 1 % sodium dodecyl sulphate solution (SDS, Sigma-Aldrich, Heidelberg, Germany) for 30 minutes on a rotating shaker at RT. Absorbance was measured at 570 nm on a Tecan spectrophotometer.

Data analysis was performed by estimating the percentage cytotoxicity by normalizing the compound concentration to that of the DMSO-treated wells. The drug concentration at which 50 %, 30 % and 10 % inhibition of cell growth was observed, were determined as IC_{50}, IC_{30} and IC_{10} values, respectively, and was calculated as described previously (35).

3.5. Assay for the inhibition of the ESC differentiation

The measurement of inhibition of ESCs differentiation was applied to predict the embryotoxic potential of the compounds. This *in-vitro* developmental toxicity test is based on the impairment of ESCs differentiation to contracting cardiomyocytes (36). EBs were formed by hanging drop method in presence of IC_{10} and IC_{30} of curcumin, EGCG, and quercetin as described above. For analysis of cell differentiation, on day 10, 160 EBs were examined for contracting myocardial foci by phase contrast microscopy and for each concentration the percentage of EBs containing spontaneously contracting cardiomyocytes was determined. Furthermore, for each concentration, 60 EBs were evaluated for the presence of contractile activity and beating frequency was determined. The results for all compound test concentrations were assessed from at least three independent experiments of which more than 60% of the EBs had differentiated into contracting myocardial cell in the solvent control.

3.6. RNA isolation and cDNA synthesis

EBs (4-, 7- and 12-days old) were collected after treatment with curcumin, EGCG and quercetin at a concentration of IC_{10} and IC_{30}. Undifferentiated ESCs and vehicle-treated EBs were collected as controls and total RNA was extracted using Trizol reagent (Invitrogen, Karlsruhe, Germany) as per the manufacturer's instructions. Genomic DNA contamination was eliminated by treating the isolated total RNA with DNaseI (Qiagen, Hilden, Germany). RNA yield and purity were assessed by spectrophotometric analysis. cDNA was synthesized by taking 2 µg total RNA and reverse transcribing with SuperScript Vilo (Invitrogen, Karlsruhe, Germany) with random hexamers according to the manufacturer's recommended protocol.

3.7. Semi-quantitative RT-PCR and quantitative RT-PCR

All PCR reactions were carried out keeping mRNA levels from beta-actin as endogenous controls. The PCR primers were designed using primer3 software (37). For semi-quantitative PCR, each reaction contained 10 µl of Dreamtaq Polymerase master mix (MBI Fermentas, St. Leon-Rot, Germany) using 0.5 µM forward and reverse primers, 1 µl of cDNA as a template in a final volume of 20 µl. The PCR reaction was carried out in a Biorad thermo-cycler with the following amplification conditions: denaturation at 95 °C/2 minutes, 22-35 cycles of 95 °C /30 sec. denaturation, 60 °C /30 sec. annealing and 72 °C /60 sec. of elongation. The PCR products were electrophoresed on 2 % agarose gels with 0.001% ethidium bromide against a 100- base pair ladder.

Real Time PCR analysis was carried out on ABI-7500 Fast PCR system (Applied Biosystems, Weiterstadt, Germany). The PCR reaction consisted of 12.5 µl SYBR Green PCR master mix (MESA-FAST mix, Eurogentec, Liege, Belgium), 1 µl of primer pair (0.4 µM each) and 1 µl of cDNA template made to a final volume of 25 µl. The standard conditions for PCR were used, 95 °C/5 minutes Taq activation, 40 cycles of 95 °C / 15 sec., 60 °C / 30sec., and 72 °C/ 30sec. A melting curve was produced to verify single PCR product amplification. The mRNA levels were normalized against beta-actin levels and calculated using a relative quantitation using the cycle threshold (ct) method by 7500 Fast System SDS software 1.4.0 (Applied Biosystems). The primer sequences used in the PCR analysis are shown in Table 1. For all PCR reactions, biological triplicates were used and reactions were carried out in triplicates for each sample. Negative controls were reactions containing H_2O as template.

3.8. Western blotting and immunocytochemistry

Expression of tissue-specific proteins during ES cell differentiation was monitored by immunoblotting and immunofluorescence staining. The following primary antibodies were used: anti-MYH6 (Abcam, Cambridge, UK), anti-ACTN1 and anti-beta-ACTIN (Sigma-Aldrich, Heidelberg, Germany). EBs (7-days and 12-days old) from vehicle control and EGCG treatment were lysed using ProteoJET lysis buffer (MBI Fermentas, St. Leon-Rot, Germany) supplemented with protease inhibitor cocktail (Sigma-Aldrich, Heidelberg, Germany). Protein content was estimated using Bradford reagent (Sigma-Aldrich, Heidelberg, Germany) and equal amounts (40 µg) of protein was resolved in a 10 % SDS-polacrylamide gel electrophoresis. Proteins were transferred to PVDF membrane (Millipore, Schwalbach, Germany). Membranes were blocked for 1 h in 5 % nonfat dry milk (Amersham, Freiburg, Germany) in TBST (10 mM Tris, 15 mM NaCl, 0.05 % Tween-20) and incubated with primary antibody overnight, washed three times for 10 minutes each, incubated in species specific secondary antibody (anti-mouse HRP or anti-rabbit HRP, Sigma-Aldrich, Heidelberg, Germany) for 1 h, washed five times for 10 minutes each, and developed using ECL detection system (Pierce Biotechnologies, Rockford, IL, USA) on photographic film (Kodak Biomax, Rochester, NY, USA).

Immunofluorescence staining was performed on trypsin-dissociated 8-days old EBs that were grown for two more days on a fibronectin coated cover slips as described previously (38). Briefly, cells growing on cover slips were rinsed twice with PBS, fixed with 4 % para-formaldehyde for 30 minutes at RT. Antibodies for the analysis of tissue-specific proteins were anti-MYH6, anti-ACTN1 for

Table 1. Primer sequences and used for RT-PCR analysis

Gene	RefSeq ID	Forward Primer	Reverse Primer
BETA-ACTIN	NM 007393	TGGTGGGAATGGGTCAGA	GTACATGGCTGGGGTGTTGA
POU5F1	NM 0136332	CAGCAGATCACTCACATCGCCA	GCCTCATACTCTTCTCGTTGGG
NANOG	NM 028016	GAACGCCTCATCAATGCCTGCA	GAATCAGGGCTGCCTTGAAGAG
SOX2	NM 011443	AACGGCAGCTACAGCATGATGC	CGAGCTGGTCATGGAGTTGTAC
TUBB3	NM 023279	CATGGACAGTGTTCGGTCTG	TGCAGGCAGTCACAATTCTC
NES	NM 016701	AGGCTGAGAACTCTCGCTTG	ATTAGGCAAGGGGGAAGAGA
PAX6	NM 013627	AGTGAATGGGCGGAGTTATG	TGAGACATGTCAGGTTCACTCC
MTAP2	NM 001039934	AGGAAAAGGCCCAAGCTAAA	GCTTGGGGACTGTGTGATG
AFP	NM 007423	TCTTCCACAAGGATCTGTGC	TGCAGCACTTCTCCAAAAGG
DCN	NM 00190451	GAGGGAACTCCACTTGGACA	CTCACAGCCGAGTAGGAAGC
SOX17	NM 011441	GCCGATGAACGCCTTTATGGTG	TCTCTGCCAAGGTCAACGCCTT
FLT1	NM 010228	CGGTTCACCTGGACTGAGAC	TCAGATTCATCGTCCTGCAC
HNF1B	NM 009330	GCCTTAGTGGAGGAGTGTAACAG	TCTGCCTGAACGCCTCTTCCTT
MYH6	NM 001164171	GCTGGAAGATGAGTGCTCAGAG	CCAGCCATCTCCTCTGTTAGGT
TBRACHUYURY	NM 009309	ACTGGTCTAGCCTCGGAGTG	GAGCCTCGAAAGAACTGAGC
BMP2	NM 007553	AGACCACCGGCTGGAGAG	GTTCCCGGAAGATCTGGAGT
ACTN1	NM 134156	TCGCCAAGTGTCAACGCTCGTT	GGTCGATGGTTTCCAGCAGCTT

cardiomyocytes and skeletal myocytes. Fixed cells were permeabilized (0.5 % Triton-X100 in PBS; 10 minutes; RT), and non-specific binding sites blocked with 5 % bovine serum albumin (Sigma-Aldrich, Heidelberg, Germany) for 1 h at RT. Thereafter cells were incubated overnight at 4 °C with (1:200) primary antibodies. Secondary antibodies (Molecular Probes, Karlsruhe, Germany) binding was done for 1 h in dark at 4 °C after thrice washing with PBS. Counter staining was done using 4', 6-diamidino-2-phenylindole (DAPI, Applichem, Darmstadt, Germany) followed by three washes with PBS. Cover slips were mounted using ProLongGold mounting medium (Molecular Probes, Karlsruhe, Germany). Images were captured with an inverted fluorescence microscope (ZeissAxiovert-200, Carl Zeiss, Oberkochen, Germany). Protein specific positive stain and DAPI counter stained cells were counted separately in five or more visual field, selected randomly. The percentage of protein specific cells was calculated according to the formula: protein positive cells / DAPI counterstained cells x 100%.

3.9. Statistical analysis

Results are shown as mean +/- SE or mean +/- SD as indicated. Statistical analysis was done using paired and unpaired Student's *t*-test where applicable. *P*-values of less than 0.05 were considered significant.

4. RESULTS

4.1. Effects of EGCG, curcumin and quercetin on ESC cytotoxicity

Figure 1A indicates the chemical structure of the compounds. All cultures started at the same initial cell density and ESCs were incubated for 72 h with 0.1 to 100 µM curcumin, quercetin and EGCG. Control wells were supplemented with either DMSO as vehicle controls or PBS as solvent control and 5-fluorouracil (5-FU) was used as a cytotoxic agent. We quantified the cytotoxicity by means of MTS and crystal violet (CV) staining assays. Figure 1B and Figure 1C shows the dose response curve for the compound tested via MTS and CV assay sets, respectively. In MTS assay, the order of cytotoxicity from high to low for the compounds was EGCG (IC_{50} = 55 +/- 5 µM), curcumin (IC_{50} = 13.2 +/- 1.5 µM), quercetin (IC_{50}= 4.2 +/- 0.3 µM) and 5-FU (IC_{50}= 1.6 +/- 0.3 µM). Figure 1D shows the morphology of the cells after treatment with the compounds at IC_{50} concentrations. As indicated treatment with quercetin resulted into markedly smaller clusters of ESCs indicating the potent toxic effects of quercetin compared to EGCG and curcumin.

4.2. Effects of the compounds on EB development

To test the effect of compounds, ESCs were differentiated using the hanging drop protocol (Figure 2A). ESCs (500 cells in 20 µl) were grown in hanging drops for 2 days in the presence or absence of the test compounds. EBs were transferred to bacteriological dishes (Figure 2A). After 4 days, cellular aggregates formed in the presence of compounds could be distinguished from control EBs in terms of morphology and size (Figure 2B). Control EBs had clear boundaries and appeared circular globular shaped (Figure 2A). As demonstrated, EGCG at concentrations used had no effects on the EB size (Figure 2B), whereas treatment with curcumin reduced the EB size. In contrast, treatment with quercetin resulted in a striking decrease of the EB size in a concentration-dependent manner, demonstrating the cytotoxic effects of quercetin.

4.3. Effects of the compounds on ESCs differentiation

Progressive differentiation of ESCs to ectodermal, endodermal and mesodermal germ layers cells and further to somatic cells was monitored by semi-quantitative PCR analysis in undifferentiated ESCs and in 4-, 7- and 12-days old EBs (Figure 3). The pluripotency marker *Pou5f1* (Oct4) was highly expressed in undifferentiated ESCs (Figure 3A), remained detectable in 4- and 7-days old EBs and was absent in 12-days old EBs, suggesting a progressive loss of pluripotency. *Pou5f1* expression was not affected by any of the three test substances (Figure 3A). Figure 3B-D shows expression

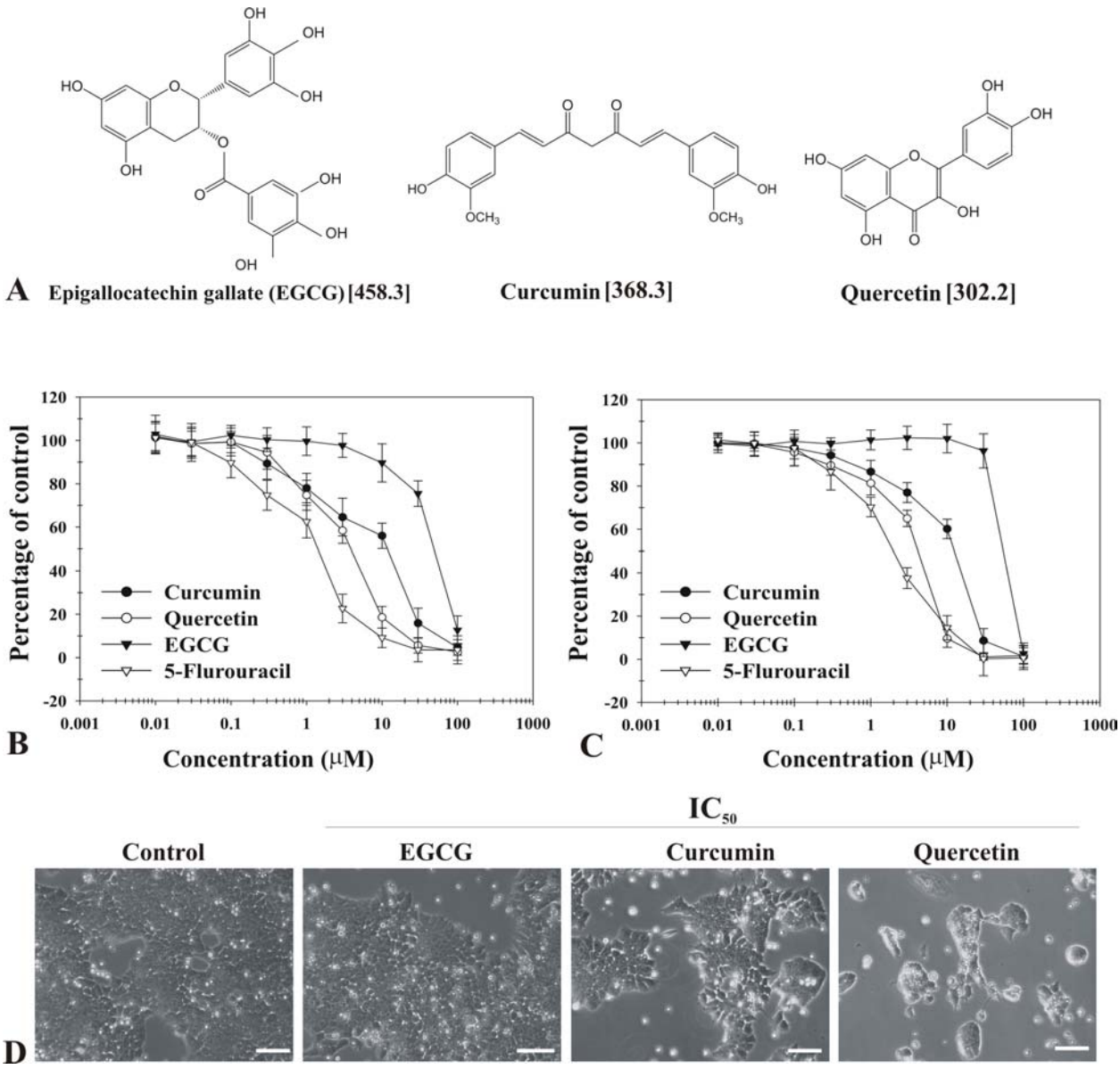

Figure 1. Effect of EGCG, curcumin and quercetin on undifferentiated and differentiating ESCs. A) Chemical structure of EGCG, curcumin and quercetin, numbers in square brackets represent molecular weight. Inhibitory effect of EGCG, curcumin, quercetin and 5FU on pluripotent CGR8 cells measured via B) MTS cytotoxicity assay, C) Crystal violet assay. D) The cytotoxic effect caused by EGCG curcumin and quercetin at IC_{50} concentration after 72 h of undifferentiated culture. Scale bar corresponds to 100 μm.

patterns of mesoderm-, ectoderm- and endoderm-associated markers. The temporal expression patterns of neuronal markers *Nes, Pax6* and *Tubb3* (Figure 3B) were not affected by the compounds at the IC_{10} and IC_{30}. As indicated in Figure 3C, the time course of the expression of the endodermal hepatic gene markers *Dcn* and alpha-fetoprotein (*Afp*), *Hnf1b* and *Flt1* was not significantly affected by the IC_{10} and IC_{30} concentrations of the test compounds. In contrast, expression of *Sox17* was inhibited in 7-days old EBs by EGCG at the IC_{30} concentration and by curcumin at IC_{10} and IC_{30} concentration. Interestingly, quercetin at IC_{30} concentration completely blocked the expression of *Sox17* even in 4-days old EBs (Figure 3C). The time course of the expression of the mesodermal markers *Tbrachyury* (early mesoderm), *Bmp2* (late mesoderm) as well as of the cardiomyocyte markers *Myh6* (alpha-myosin heavy chain) and *Actn1* (alpha-cardiac actinin) was not affected after treatment of ESCs with the various compounds (Figure 3D).

For more accurate quantification of mRNA levels, we applied qRT-PCR methodology. Treatment of the cells with all three compounds did not influence the expression of the pluripotency markers *Pou5f1, Nanog* and

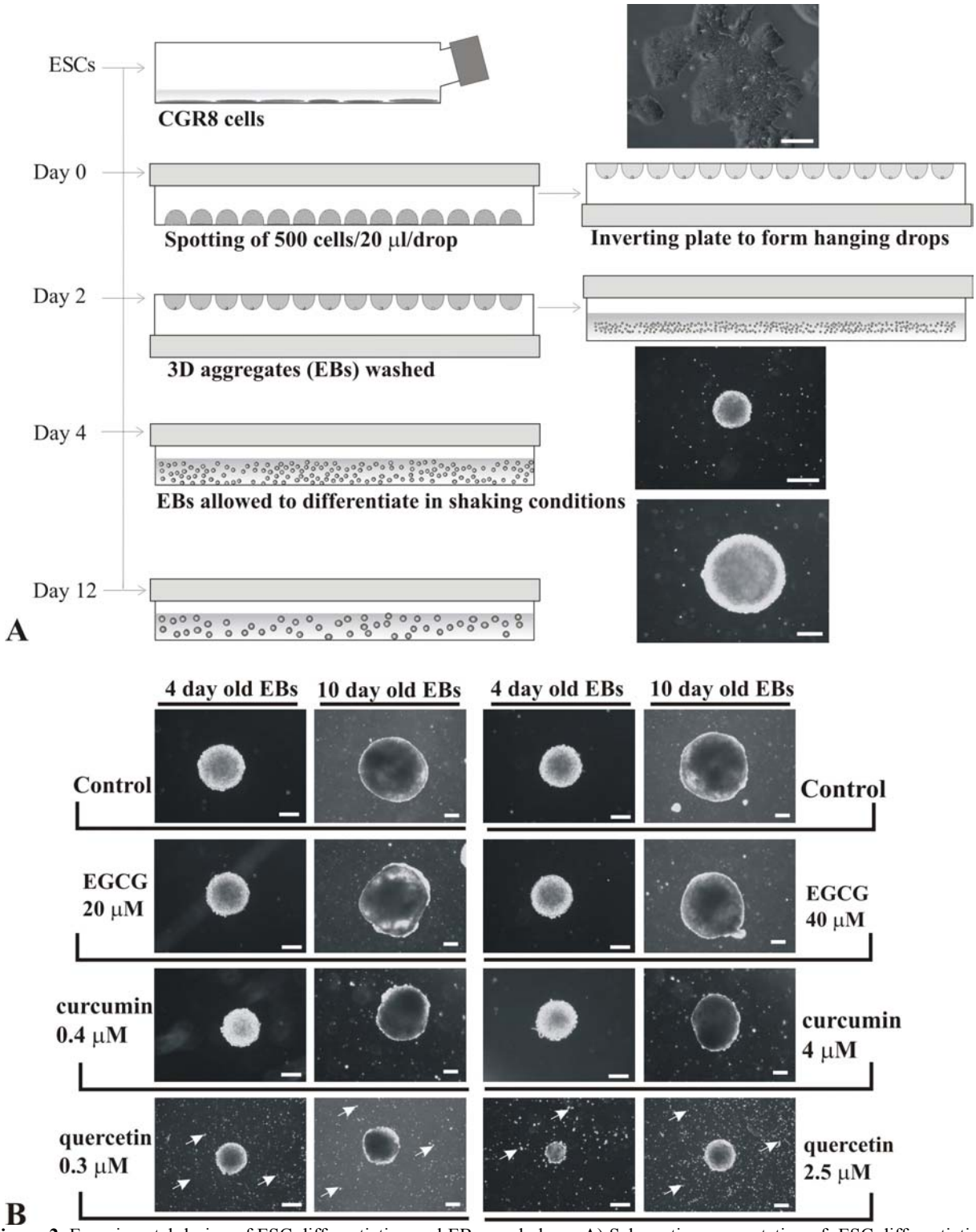

Figure 2. Experimental design of ESC differentiation and EB morphology. A) Schematic representation of ESC differentiation using hanging drop method. ESCs are dissociated and resuspended in differentiation medium and hanging drops of 500 cells / 20 µl are made on low-adherent plates. Hanging drops allow cells to aggregate in 3 dimensional structures called EBs, which initiates ESC differentiation. The EBs grow in size, maintaining the spherical structure, and form cells of the three germ layers, including functional cardiomyocytes which are observed as spontaneously beating foci in EBs. B) 4 and 10 day old EBs treated with IC_{10} and IC_{30} concentrations of EGCG, curcumin and quercetin. Arrow head represents dead cells. Scale bar corresponds to 100 µm.

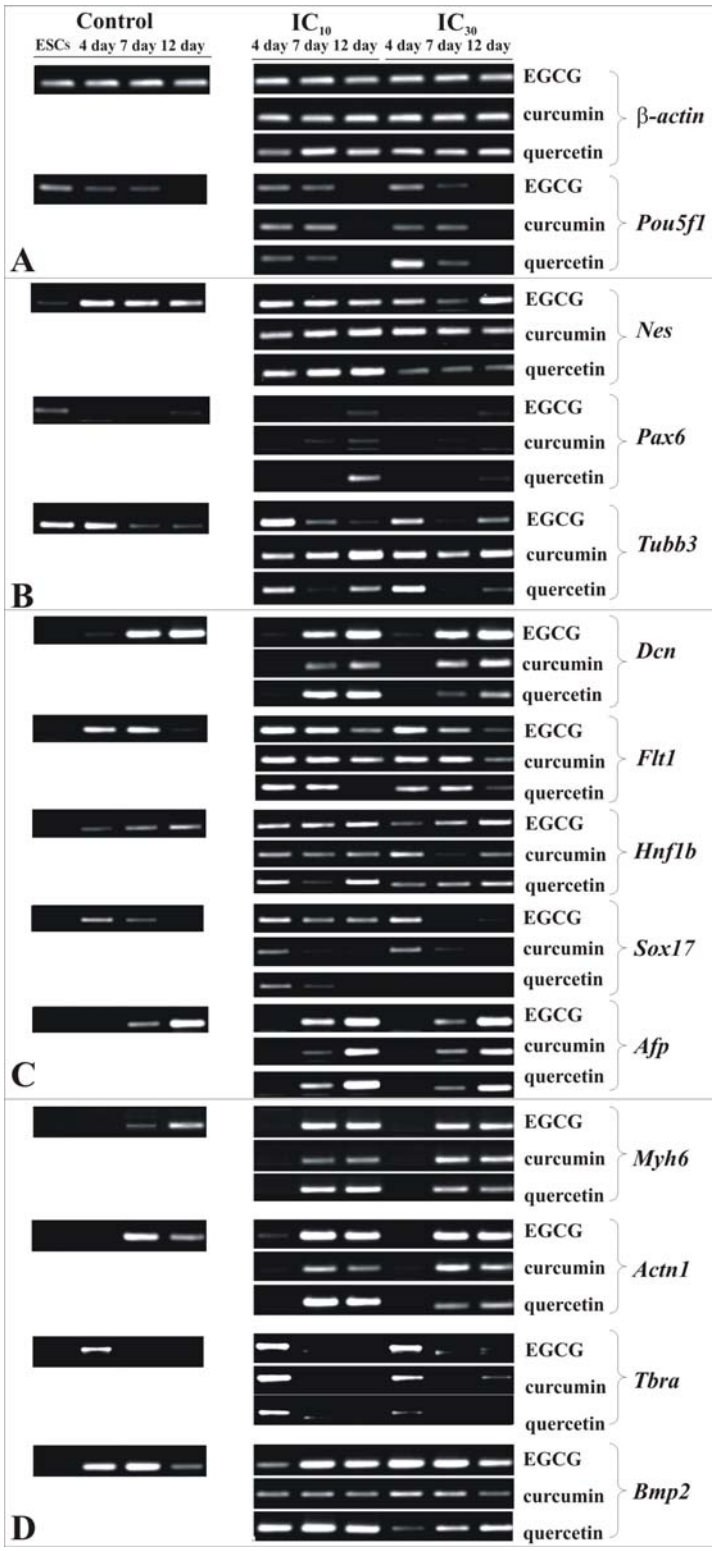

Figure 3. Effect of EGCG, curcumin and quercetin on ESC differentiation. ESCs were allowed to differentiate in presence of compounds at IC_{10} and IC_{30} concentrations. After 4, 7 and 12 days of differentiation, total RNA was extracted and synthesised cDNA was used for gene expression profiling by semi-quantitative RT-PCR. Pluripotency and differentiation markers were examined. A) house-keeping control *beta-actin* and pluripotency marker *Pou5f1*, and characteristic ESC differentiation markers of B) ectoderm (*Nes, Pax6, Tubb3*), C) endoderm (*Dcn, Flt1, Hnf1b, Sox17, Afp*) and D) mesoderm (*Myh6, Actn1, Tbrachyury, Bmp2*). Control sample was treated with vehicle.

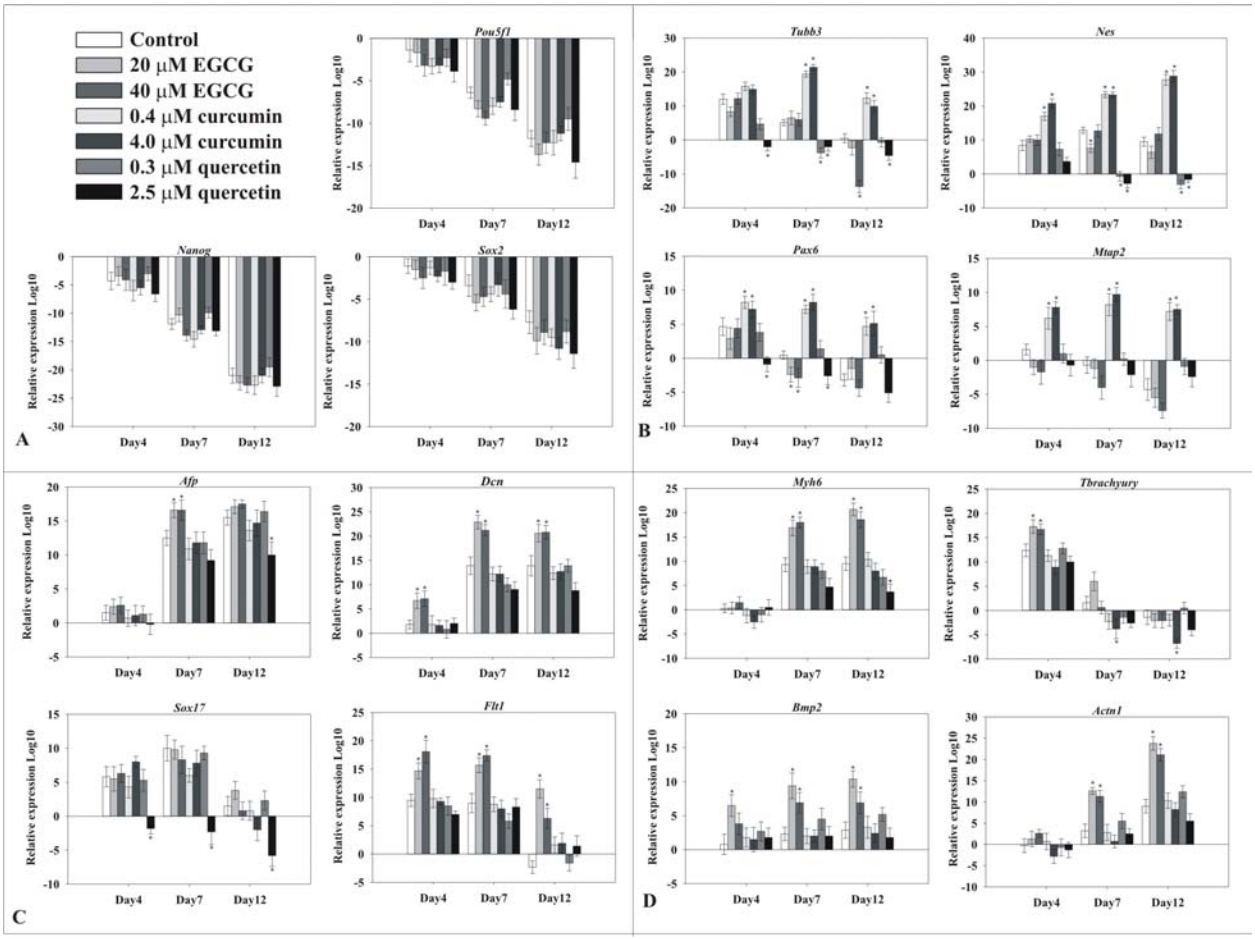

Figure 4. Gene expression of ECS differentiation affected by compound treatments. ESCs differentiated into EBs in the presence of EGCG, curcumin and quercetin at IC_{10} and IC_{30} concentrations were characterised at transcript levels using quantitative RT-PCR. The study was performed from 4-, 7- and 12- days of EBs and normalized to gene expression in undifferentiated ESCs represented as relative quantitation. A) represents the pluripotency markers *Pou5f1 Nanog and Sox2*, B) ectodermal (*Mtap2, Nes, Pax6, Tubb3*), C) endodermal (*Dcn, Flt1, Sox17, Afp*) and D) mesodermal markers (*Myh6, Actn1, Tbrachyury, Bmp2*). Bar denotes relative gene expression in $\log_{10} \pm$ SD. Asterix implies $P < 0.05$

Sox2 (Figure 4A). Compared to the control EBs, the expression of *Nes* in 7- and 12-days old EBs was significantly inhibited after treatment with quercetin whereas treatment with curcumin resulted in an elevated expression level of *Nes* (Figure 4B). Curcumin treatment resulted in an elevation of the expression level of *Nes* in 4-, 7-, and 12-days old EBs, whereas EGCG treatment did not have an effect on the gene expression of *Nes*. *Pax6* expression was enhanced in the 4-, 7-, and 12-days old curcumin-treated EBs compared to the control EBs. Curcumin also elevated the expression of *Tubb3* in 7- and 12-days old EBs whereas 40 μM EGCG and 2.5 μM quercetin significantly inhibited the *Tubb3* expression compared to the control EBs (Figure 4B). *Mtap2* expression in 4-, 7- and 12- days old EBs was increased by curcumin, but was downregulated by EGCG and quercetin treatment (Figure 4B). Endodermal markers *Afp, Dcn*, and *Flt1* were upregulated significantly in 4-, 7- and 10-day old EBs treated with both concentrations of EGCG. Quercetin inhibited *Afp* expression in 12-day old EBs, whereas quercetin and curcumin did not significantly alter *Dcn* or *Flt1 expression* (Figure 4C). The endoderm-specific marker *Sox17* was not affected by EGCG or curcumin treatments, while quercetin significantly down regulated the *Sox17* expression for 4-, 7- and 12- days old EB treated at IC_{30} concentrations (Figure 4C). No significant effects of EGCG on the expression of *Myh6* and *Actn1* were observed in 4-days old EGCG-treated EBs, compared to control EBs (Figure 4D). However, an increase of *Bmp2* and *Tbrachyury* expression has been observed in the 4-days old EGCG-treated EBs. Interestingly, a significant increase of *Myh6, Bmp2* and *Actn1* expression in 7- and 10-days EGCG-treated EBs (at 20 and 40 μM) was observed (Figure 4D). Curcumin had no effect on *Myh6*, *Bmp2* and *Actn1* expression, however the mesodermal transcription factor *T (brachyury)* was significantly downregulated by higher concentration in 7- and 12-days old EBs. Quercetin caused no significant change in expression of mesodermal markers (Figure 4D).

4.4. Effect of EGCG on the differentiation of ESCs into cardiomyocytes

Since an increased expression of the *Myh6* and *Actn1* was observed after treatment of the cells with EGCG, the effects of EGCG in non-cytotoxic concentrations of 20 and 40 µM on the beating activity of spontaneously differentiated cardiomyocytes generated from ESCs applying the protocol as described in Figure 2A.

Each EB contained one or more beating areas and an increase in size strength of contraction and beating frequency was observed in EGCG-treated EBs during further differentiation steps (supplementary video v1). In control EBs, 64 % of the total 12-days old EBs contained beating areas (n = 160, Figure 5A). The percentage of 12-days old beating EBs was significantly increased by 20 and 40 µM EGCG to 81 % and 72 %, respectively (Figure 5A). Cardiac differentiation of EBs treated with 20 and 40 µM of EGCG was enhanced in comparison to control 10- and 12-days old EBs. In contrast to EGCG, quercetin at both concentrations markedly reduced the number of the 10 and 12-days old beating EBs whereas curcumin slightly (but not significantly) reduced the number of beating EBs. Longer culture times resulted in cell death and decrease of beating EBs. To test whether the observed increased cardiomyogenic effect of EGCG correlates with an enhanced expression of cardiomyocyte-specific cytoskeletal proteins, immunostainings of alpha-cardiac-actinin (ACTN1) and alpha-Myosin heavy chain (MYH6) were done for control and treated EBs. The beating cardiomyocytes cells stained positively with anti-ACTN1 mAb and anti- MYH6 mAB (Figure 5B). The percentage of alpha-actinin positive cells was determined.

This quantitative assessment further verified these structural findings, on 10- day, only 12 % percent cells stained positively for ACTN1 in the control mounts, whereas 20 µM EGCG and 40 µM EGCG yielded 23 % and 18 % cells respectively were positively stained for cardiomyocytes. In addition, the proportion of cardiomyocytes induced by 20 µM of EGCG that contained MYH6 positive labeled areas was significantly increased by about 18 % in contrast with the case of 9 % in the control. In addition, treatment of EBs with 20 and 40 µM EGCG also resulted in remarkable increase in the beating frequency of EBs of spontaneously beating cardiomyocytes by a factor of 9 % and 10 % respectively (Figure 5C, supplementary video 1). Moreover the protein expression measured by Western blotting for cardiac muscle protein MYH6 were detected to be expressed in significantly higher amounts in 7-, and 12-day EBs treated with EGCG compared to the controls (Figure 5D).

5. DISCUSSION

Of the substances tested, quercetin was the most cytotoxic among the three compounds and inhibited the expression of ectoderm- and endoderm-related genes. Curcumin was less cytotoxic than quercetin and enhanced the expression of neural genes during the differentiation of ESCs. Curcumin had no effect on the cardiomyogenic differentiation of ESCs whereas quercetin even inhibited cardiomyogenesis. Our study demonstrates that in contrast to curcumin and quercetin, EGCG is cytotoxic to undifferentiated ESCs only in high concentrations. Non-cytotoxic concentrations of EGCG induced an increase of expression of cardiomyocyte-specific genes such as *Myh6* and *Actn1*. This gradual increase corresponded to an increase in protein expression MYH6 and ACTN1 as measured by Western blotting and/or immunocytochemistry after treatment with 20 µM and 40 µM of EGCG and further confirms the stimulation of cardiac differentiation. Accordingly, the number of beating EBs and their beating frequency was elevated. Gene expression analysis measured by qRT-PCR revealed increased expression of mesodermal transcription factors and structural proteins. Some endodermal markers that were part of either early endoderm or meso-endoderm were also upregulated. However, ectoderm markers were for the most part not significantly affected by the EGCG treatments. It was previously shown that stimulation of cardiomyogenesis by exogenous ROS not only increases the number of beating cardiomyocyte foci, but also enhances the expression of cardiac-specific genes and transcription factors (39). Interestingly, radical scavengers can increase the level of cardiac gene expression, which indicates that these factors are regulated by redox-changes rather than absolute increase or decrease of intracellular ROS. Differentiating ESCs are capable of endogenously generating ROS (40, 41). In an independent report, EGCG alone induced production of H2O2 in a concentration-dependant manner, and activates AMPK through the production of ROS (42). In our previous study, we tested EGCG at entirely different conditions including the concentrations tested (43)., EGCG was added to 1-day old murine EBs instead of directly to undifferentiated ESCs prior to EB formation. Under these conditions at 5 µM we found a slight inhibition of cardiogenesis. These results emphasize that the experimental conditions are of decisive importance for screening of molecules for specific cell differentiation. In this context, it should be considered that ESC differentiation is a dynamic process including the differential expression of numerous genes and gene products that could be targets of EGCG. Also, the test concentration of EGCG has a particular importance. Several studies demonstrated that EGCG inhibits biological signaling such as mitogen activated protein kinase, cyclin-dependant kinase, growth factor-related cell signaling, PDGF-BB, nuclear factor kB and topoisomerase I at a concentration higher than 20 µM (42, 44). Another publication reports that green tea polyphenols protect against myocardial damage and enhance cardiac contractility by modulating myofilament Ca^{2+} sensitivity in post-MI rats. It was shown that EGCG acts by modulating pH-induced changes in myofilament Ca^{2+} sensitivity in cardiac muscle by binding to cardiac troponin-C (45, 46). The mESC model described here examines key developmentally regulated genes affected by developmental toxicants with a fast turn around time and reduced costs, and with certain modification, could be used with human ESCs to better predict the effects on embryo development.

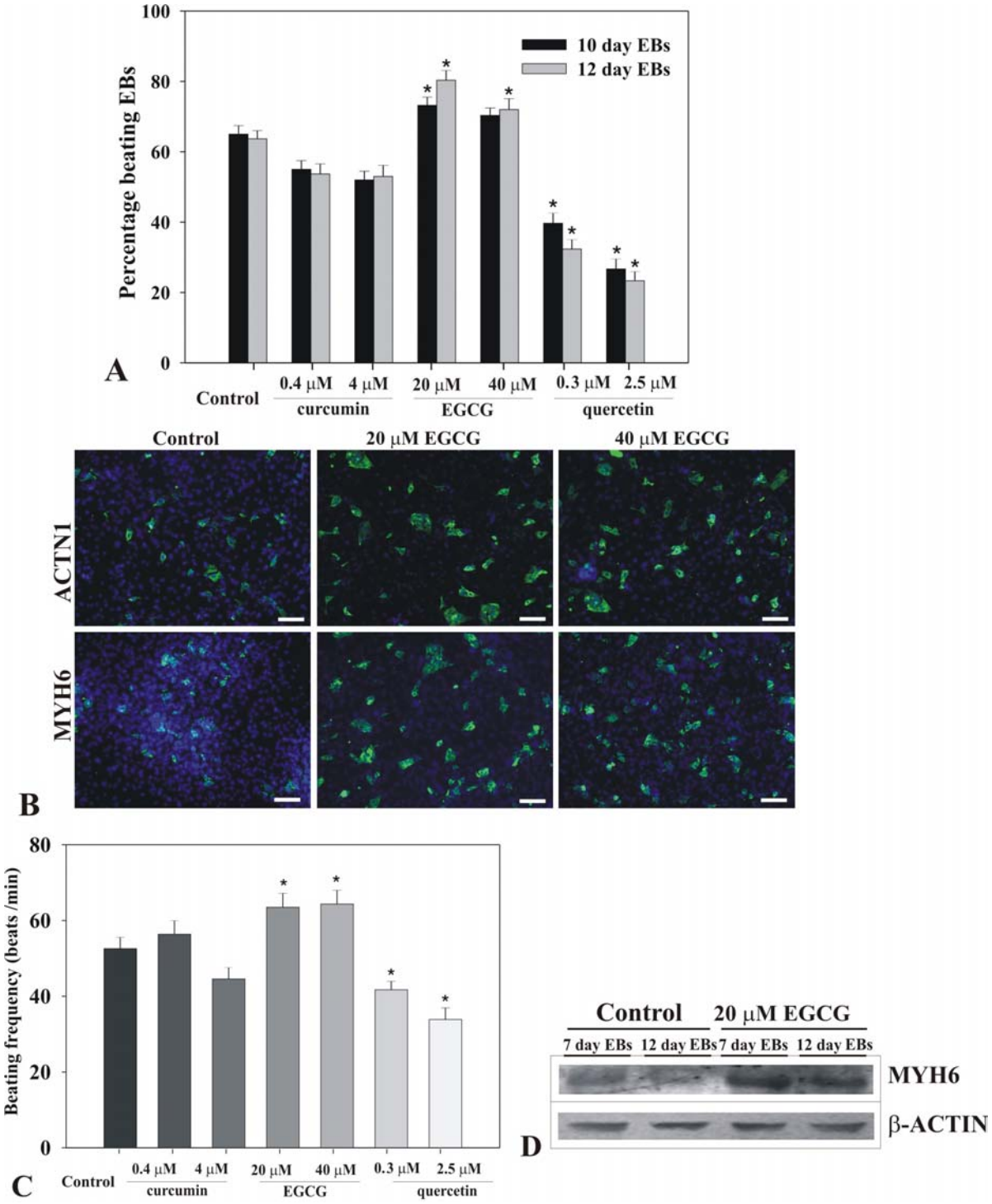

Figure 5. Effect of EGCG, curcumin and quercetin on ESC differentiation into beating EBs. ESCs differentiated into EBs in presence of IC_{10} and IC_{30} concentrations were evaluated for the beating abilities. A) Percent differentiation into beating EBs counted on day 10 and 12 day after treatments. The percentage was determined by counting 160 EBs in three biological replicates. Bar represents percent beating EBs ± SE. Immunostaining for Bi) ACTN1 and Bii) MYH6 done from 10 day old differentiating cells treated with IC_{10} and IC_{30} concentrations of EGCG, Scale bar corresponds to 100 μm. C) Estimation of spontaneous beating frequency for compound treatments. Sixty EBs were analyzed for measuring beating frequency, represented as a bar of beats per minute ± SE. and D) Cardiac specific protein (MYH6) expression on 7 day and 12 day old EBs treated with EGCG.

Curcumin is a natural phenolic compound and has been reported to be capable of scavenging free radicals and inhibiting inflammation (47-49), contributing to cancer chemoprevention by tumor growth suppression (8, 50, 51). Results of animal studies suggest that curcumin may also be beneficial in neurodegenerative conditions such as Alzheimer's disease (52-54), focal cerebral ischemia (55-58) and has a protective role against certain injuries to hippocampus neurons (59, 60). In our study, curcumin-treated EBs were virtually indistinguishable from control EBs in terms of morphology. We found that curcumin significantly increased the differentiation of ESCs towards ectodermal lineages, suppressing the other lineages to some extent. However, the elevated expression of neuronal gene markers due to curcumin treatment remains to be elucidated.

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Abbreviations: MI- myocardial infarction; COX-2-cyclooxygenase-2; MMP-9- matrix metalloproteinase-9; DNA-deoxyribonucleic acid; H_2O_2-hydrogen peroxide; EGCG- (-)-epigallocatechin-3-gallate; CVD-cardiovascular diseases; PDGF- platelet derived growth factor; PDGF-R- platelet derived growth factor-receptor; ROS- reactive oxidative species; CHD- coronary heart disease; HSP70- heat shock protein 70;

Key Words: Chemoprevention, Epigallocatechin-3-O-gallate, Quercetin, Curcumin, Embryonic Stem Cells, Embryonic Stem Cell Differentiation

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