Tropical Journal of Pharmaceutical Research May 2014; 13 (5): 689-696
**ISSN:** 1596-5996 (print); 1596-9827 (electronic)


Available online at http://www.tjpr.org
**http://dx.doi.org/10.4314/tjpr.v13i5.6**

**Original Research Article**

# Assessment of the Developmental Toxicity of Epidermal Growth Factor using Embryonic Stem Cell Test

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*Received: 8 February 2014* *Revised accepted: 22 April 2014*

## Abstract

***Purpose:*** *To determine whether epidermal growth factor (EGF) is involved in reproductive developmental toxicity, using the embryonic stem cell test (EST), as well as ascertain how EGF influences embryonic development.*

***Methods:*** *To predict developmental toxicity on the basis of reducing cell viability and inhibition of differentiation of embryonic stem cells, EST was used to assess changes in different blastodermic genes and expression of proteins including ectodermal-specific genes Pax6, NF-H and glial fibrillary acidic protein (GFAP), mesodermal-specific genes BMP4, GATA4, and MyoD, and endodermal-specific genes, viz, α-fetoprotein, transthyretin (TTR), and albumin, as well as undifferentiated genes, Nanog and Oct4.*

***Results:*** *The results indicate that EGF was weakly embryotoxic with $IC_{50}$ESC (i.e., the concentration that reduced ESC viability by 50 %), $IC_{50}$3T3 (the concentration that reduced 3T3 cell viability by 50 %), and $ID_{50}$ESC (the concentration that inhibited differentiation of ESC by 50 %) of 6.773, 10.531, and 1.793 μg/mL, respectively. The expression levels of tissue-specific genes of the three germ layers were mainly promoted by 0.01 - 1 μg/mL EGF. Distinctively, relatively high concentrations of EGF caused a discordant effect on the three germ layers. High concentrations of EGF promoted differentiation of the ectoderm and mesoderm, and either inhibited or had mostly no impact on the endoderm.*

***Conclusion:*** *The imbalance of the three layer-specific genes and expression of proteins, as a result of EGF, might be responsible for its weak level of developmental toxicity. The sensitivity of TTR means that further investigation is required to determine whether it can be used as an embryotoxicity biomarker for growth factors.*

***Keywords:*** *Embryonic stem cell test, Epidermal growth factor, Developmental toxicity, Germ layers, Blastodermic genes, Protein expression*

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## INTRODUCTION

Growth factors, including acidic fibroblast growth factor (aFGF), basic FGF (bFGF), nerve growth factor (NGF), epidermal growth factor (EGF), etc., are naturally occurring substances that are capable of stimulating cellular *growth*, proliferation and cellular differentiation, and also regulate a variety of cellular processes. In addition, they play an important role in the process of embryonic development [1]. For example, NGF may be of critical importance for ovarian follicles [2]. Insulin-like growth factor (IGF-I) participates in the regulation of fetal growth and development, and an abnormal level of IGF-I might be responsible for intrauterine growth retardation (IUGR) or large for date

infants [3]. EGF promotes the proliferation, survival, and regeneration of multiple cell types, especially those of the ectoderm and mesoderm [4]. Previous research showed that abnormal expression of EGF and EGF receptor affected the normal development of the embryo, and that the serum and urine concentrations of EGF were significantly higher in infertile women than in a normal control group [5]. It is certain that EGF plays an important role in improving the rate of hatched blastocysts and reducing the apoptosis in vitro; when EGF concentration reached to 0.1 μg/mL, not only did the apoptosis and necrosis rate of embryonic cells significantly increase, but also embryonic development was inhibited [6]. Therefore, it is worth examining whether EGF has potentially toxic effects when used in a specific population, such as women in early pregnancy for a long time or at a large dose.

In the present study, we firstly evaluated the embryotoxic potential of EGF *in vitro* using a classic embryonic stem cell test (EST) protocol, and further investigated the effects of EGF on the developmental process. Embryotoxicity was determined by the three endpoints defined in the EST, which are $IC_{50}$ESC, $IC_{50}$3T3, and $ID_{50}$ESC. The embryotoxic mechanism of EGF was studied by detecting the expression of tissue-specific genes in three germ layers; ectodermal-specific genes NF-H, glial fibrillary acidic protein (GFAP), and Pax6, mesodermal-specific genes GATA4, MyoD, and BMP4, endodermal-specific genes α-fetoprotein (AFP), transthyretin (TTR) and albumin (ALB), as well as undifferentiated genes Nanog and Oct4. Combining this with the detection of tissue-specific proteins TTR, BMP4, and NF-H, we comprehensively investigated the way in which EGF affected embryonic development.

## EXPERIMENTAL

### Cell culture

Cells of the mouse embryonic stem (ES) cell line $OG_2$ were purchased from Guangzhou Institute of Biomedicine and Health. Mouse embryonic fibroblasts (MEFs), which were extracted from 13.5 days post-coitum fetuses from Kun-ming mice, as the feeder layer, were inactivated with 10 μg/mL of mitomycin (Santa) for 3 h. The ES cells were passaged on MEFs cultured on gelatin (0.1%, Sigma)-coated plates, maintained in KnockOut™ Dulbecco's modified Eagle's medium (KnockOut DMEM; Gibco) at 37°C and 5 % $CO_2$. The medium was supplemented with 15% KnockOut™ Serum Replacement (KSR; Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin, 1% non-essential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Sigma), and 1000 U/mL leukemia inhibitory factor (LIF; Millipore) to maintain their undifferentiated status. The medium was refreshed every day and ES cells were passaged every 3-4 days. The differentiation medium consisted of 15% fetal bovine serum (FBS; Gibco), instead of KSR, and an absence of LIF for complete development.

The Balb/c 3T3 cell line was purchased from the cell bank of Zhongshan Medical University, cultured in DMEM (Gibco) supplemented with 10% FBS (HyClone), 100 U/mL penicillin, and 100 U/mL streptomycin, and passaged every 2-3 days.

### Cytotoxicity measurement

The cytotoxicity assay was performed as described [7], the cytotoxic effects of EGF on 3T3 and ES cells were determined by CCK8. On day 1, 500 cells per 200 μL medium were seeded in a 96-well plate and cultivated with a series of different concentrations of EGF for 10 days. The medium was renewed on days 3 and 5. On day 10, after incubating the cells with CCK8 solution (Sigma) for 1-3 h at 37°C, absorbance was measured by a spectrophotometer at 450 nm. The cytotoxicity of the test substance, expressed as the concentration that reduced the cell viability by 50% in comparison with control ($IC_{50}$), was determined from a concentration-response curve.

### ESC differentiation assay

As previously described [8], 1000 cells in a droplet of 20 μL medium were in a culture on the inner lid of a dish in a “hanging drop” to allow differentiation. During this period, the cells aggregated and became embryoid bodies (EBs), which are frequently used to facilitate differentiation toward cell types of all three germ layers [9] . After culturing for 3 days, the EBs were transferred to dishes containing EGF for another 2 days. On day 5, the EBs were seeded on 0.1% gelatin-coated 6-well plates and incubated for an additional 5 days for complete differentiation. The $ID_{50}$ of EGF, expressed as the concentration that suppressed the expression of β-MHC by 50% in comparison with control, was calculated from a concentration-response curve.

### Classification of the embryotoxic potential of EGF

According to the guidelines of European Centre for the Validation of Alternative Methods

**Table 1:** Primer sequences used for Q-PCR

| Genes | Sequences (5′-3′) Forward | Sequences (3′-5′) Reverse |
|---|---|---|
| GAPDH | CCTTCCGTGTTCCTACCC | CCCAAGATGCCCTTCAGT |
| β-MHC | GCCCTTTGACCTCAAGAAAG | CTTCACAGTCACCGTCTTGC |
| Oct4 | GGTGGAGGAAGCCGACAAC | TTCGGGCACTTCAGAAACATG |
| Nanog | CTCAAGTCCTGAGGCTGACA | TGAAACCTGTCCTTGAGTGC |
| ALB | CAGCAATGGCAGGCAGATC | GGAACTTGCCAAGTACATGTGTGA |
| AFP | CCTCAGCAGAGCTGATCGAC | AAAATGTCGGCCATTCCCTCA |
| TTR | GTCCTCTGATGGTCAAAGTC | TCCAGTTCTACTCTGTACAC |
| BMP4 | CTGCCGTCGCCATTCACTAT | TGGCATGGTTGGTTGAGTTG |
| GATA4 | CTGGAGGCGAGATGG | GGTGGTGGTAGTCTGG |
| MyoD | ACGGCTCTCTCTGCTCCTTTG | CGTGCTCCTCCGGTTTCA |
| GFAP | TGCCACGCTTCTCCTTGTCT | GCTAGCAAAGCGGTCATTGAG |
| NF-H | GCAGGAGGAGTGCGGCTAC | CCAACCTCACTCGGAACCACT |
| Pax6 | CCGCCCTCACCAACACGTACAGT | TTGCATGTGCGGAGGGGTGTAG |

(ECVAM), combined with the three endpoints $IC_{50}$ESC, $IC_{50}$3T3 and $ID_{50}$ESC, the test compound was classified into three categories as follows: non- embryotoxic (Class I), weakly embryotoxic (Class II) and strongly embryotoxic (Class III). Three endpoints obtained in each experiment were used to calculate Functions I, II, and III as shown below [10]:

I. 5.9157 lg($IC_{50}$3T3) + 3.500 lg($IC_{50}$ES) - 5.307 ($IC_{50}$3T3 - $ID_{50}$ES)/ $IC_{50}$3T3 - 15.72

II. 3.6511 lg ($IC_{50}$3T3) + 2.3941 lg ($IC_{50}$ES) - 2.033 ($IC_{50}$3T3 - $ID_{50}$ES) / $IC_{50}$3T3 - 6.85

III.-0.125 lg ($IC_{50}$3T3) - 1.917 lg ($IC_{50}$ES) + 1.500 ($IC_{50}$3T3 - $ID_{50}$ES) / $IC_{50}$3T3 - 2.67

The classification criteria were as follows: Class I: if I>II and I>III; Class II: if II>I and II>III; and Class III: if III>I and III>II.

### Real -time quantitative PCR (Q-PCR) analysis

To verify the effect of EGF treatment on ES cells, the expression levels of ectoderm-specific genes (GFAP, NF-H and Pax6), mesoderm-specific genes (BMP4, GATA4 and MyoD), and endoderm-specific markers (AFP, TTR, and ALB) were analyzed by Q-PCR. Total RNA was extracted using an RNA extraction reagent kit (Sigma). The amount of RNA (1 µg) required to reverse transcribe was quantified by PrimeScript® 1st Strand cDNA Synthesis Kit (TAKARA), according to standard protocol, in a volume of 20 µL. Q-PCR was carried out using a SoAdvanced™ SYBR® Green (Bio-rad), according to protocol is in a final volume of 20 µL. The primer sequences are summarized in Table 1. The Q-PCR amplification protocol was as follows: 10 min at 95°C followed by 40 cycles at 95°C for 30 s and 60°C for 20 s. GAPDH used as a reference gene to analyze the tissue-specific gene quantitatively.

### Western blot

On day 10 after differentiation, the EBs were collected and extracted using protein lysate. The protein concentration was determined by a bicinchoninic acid protein assay kit (Thermo). After clearing by centrifugation at 12,000 g for 40 min, the lysate was incubated with loading buffer and heat denatured for 10 min. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane under a constant current, following which the membrane was blocked with 5% bovine serum albumin for 1 hour and incubated with the appropriate primary and secondary antibodies. Proteins were detected using enhanced chemiluminescence by ECL Western blot Kit (Bio-rad). GAPDH used as a reference gene to analyze tissue-specific protein quantitatively.

### Quantification and statistical analysis

Q-PCR analysis and Western blots were quantified by densitometry using Bio-rad CFX Manager and ImageJ 1.44, respectively. All statistical analyses and graphs were prepared using GraphPad Prism 5 software. Data were analyzed by two-way analysis of variance coupled with a Bonferroni *post hoc* test or t test, and $p < 0.05$ was considered as statistically significant.

## RESULTS

### Cytotoxic effects of EGF

The cell viability assay was used to assess the cytotoxic effect of EGF on ES cells and Balbc-3T3 fibroblasts, representing embryonic tissues and adult tissues, respectively. The results showed that, at a concentration range of 0.01 -

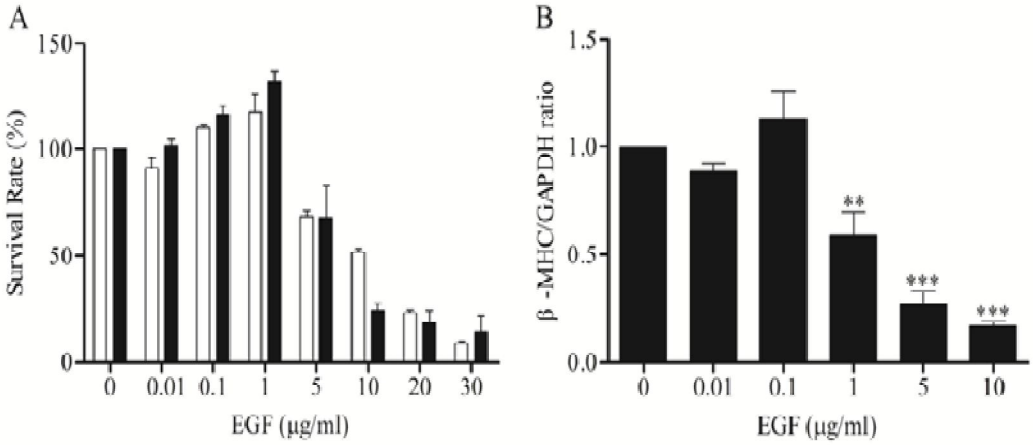


**Fig 1:** Effect of EGF on cytotoxicity and ES cell differentiation. (A) Cytotoxicity assay of EGF on ES cells and Balbc-3T3 fibroblasts (white column: Balbc-3T3; black column: ES cells). (B) β-MHC expressions of EBs cultivated in the presence of EGF detected by Q-PCR. Data presented are the mean O.D. values (±S.D.) of triplicate samples; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control sample.

30 μg/mL EGF induced or inhibited proliferation of the two cell lines at different levels (Fig 1A). EGF promoted ES cell proliferation at concentrations under 1 μg/mL, while it inhibited cell survival when its concentration was higher than 1 μg/mL. ES cells were more sensitive to cytotoxicity than Balbc-3T3 fibroblasts. $IC_{50}$ concentrations, the inhibitory concentrations of 50 % cell viability, were calculated at 6.773 μg/mL and 10.531 μg/mL for ES cells and Balbc-3T3 fibroblasts, respectively.

**Effect of EGF on ES cell differentiation**

To characterize the effects of EGF on ES cell differentiation, the sample that was extracted from the EBs was detected by Q-PCR in order to determine the expression levels of differentiation of specific gene β-MHC (Fig. 1B). The expression levels of β-MHC decreased except at the dose 0.1 μg/mL dose, showing that high doses of EGF inhibited the differentiation of ES cells. $ID_{50}$, the concentration that suppressed the expression of β-MHC by 50%, was calculated from a concentration-response curve, and was 1.793 μg/mL.

**Embryotoxicity of EGF**

According to the principle of ECVAM, combined with the three endpoints $IC_{50}$ESC, $IC_{50}$3T3 and $ID_{50}$ESC, which when calculated were 6.773 μg/ml, 10.531 μg/ml and 1.793 μg/ml, respectively, EGF was classified as weakly embryotoxic because of its II >I and II > III.

**Effects of EGF on the expression of three germ layer-specific markers**

To characterize the effects of EGF on the ES cell differentiation, the expression levels of tissue-specific genes and proteins were examined with EBs exposed to different concentrations of EGF on day 10 of differentiation. The results showed that the expression levels of undifferentiated genes Nanog and Oct4 increased in a dose-dependent manner (Fig 2), which clearly showed that EGF impeded the differentiation of ES cells.

In the endoderm lineages, AFP and ALB, which are widely used as hepatic progenitor cell markers, increased with concentrations of up to 1 μg/mL EGF, and then decreased. As shown in Fig 3, overall, mRNA expression of the endodermal marker, TTR, was declined in a dose-dependent manner. To assess the effect of EGF on differentiation of ES cells into the mesoderm lineage, we investigated mRNA expression of BMP4, cardiac-specific GATA4, and MyoD (Fig. 3). The expression level of the three genes was promoted by EGF at concentrations of 0.01-5 μg/mL, but was reduced at a concentration of 10 μg/ml. With regard to ectoderm lineages, GFAP, a representative astrocyte-specific marker, and NF-H and Pax6, which are later-stage neuron-specific markers, the expression showed the same tendency, increasing from 0.01-5 μg/mL (Fig 3).

The changes in the protein of EBs that were treated with 0.01 - 10 μg/mL EGF were detected via Western blot assay with three antibodies against TTR, BMP4, and NF-H, which are from the three germ layers, respectively (Fig 4).

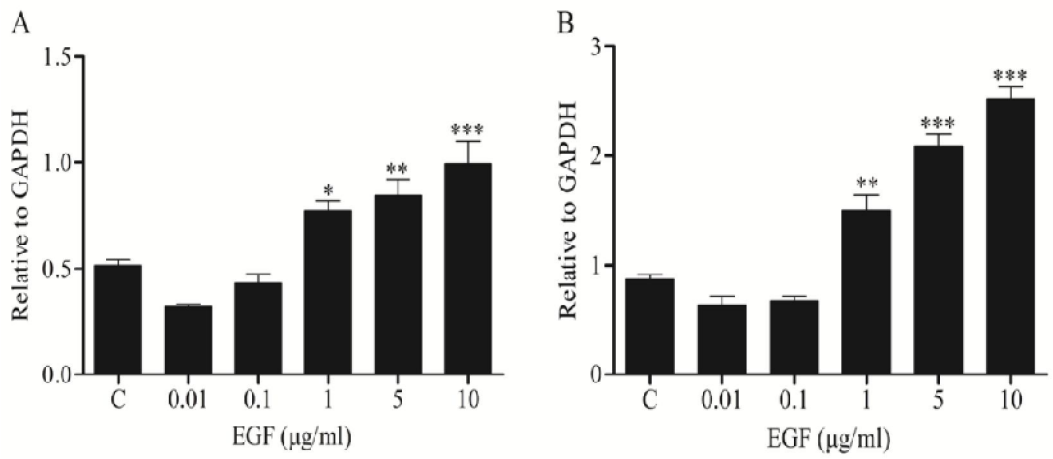


**Fig 2:** Effects of EGF on the expressions of undifferentiated genes Nanog and Oct4. (A-B) The expression levels of Nanog and Oct4 in EBs under corresponding concentrations of EGF. The data represented the mean of three independent experiments ± standard deviation; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the control sample.

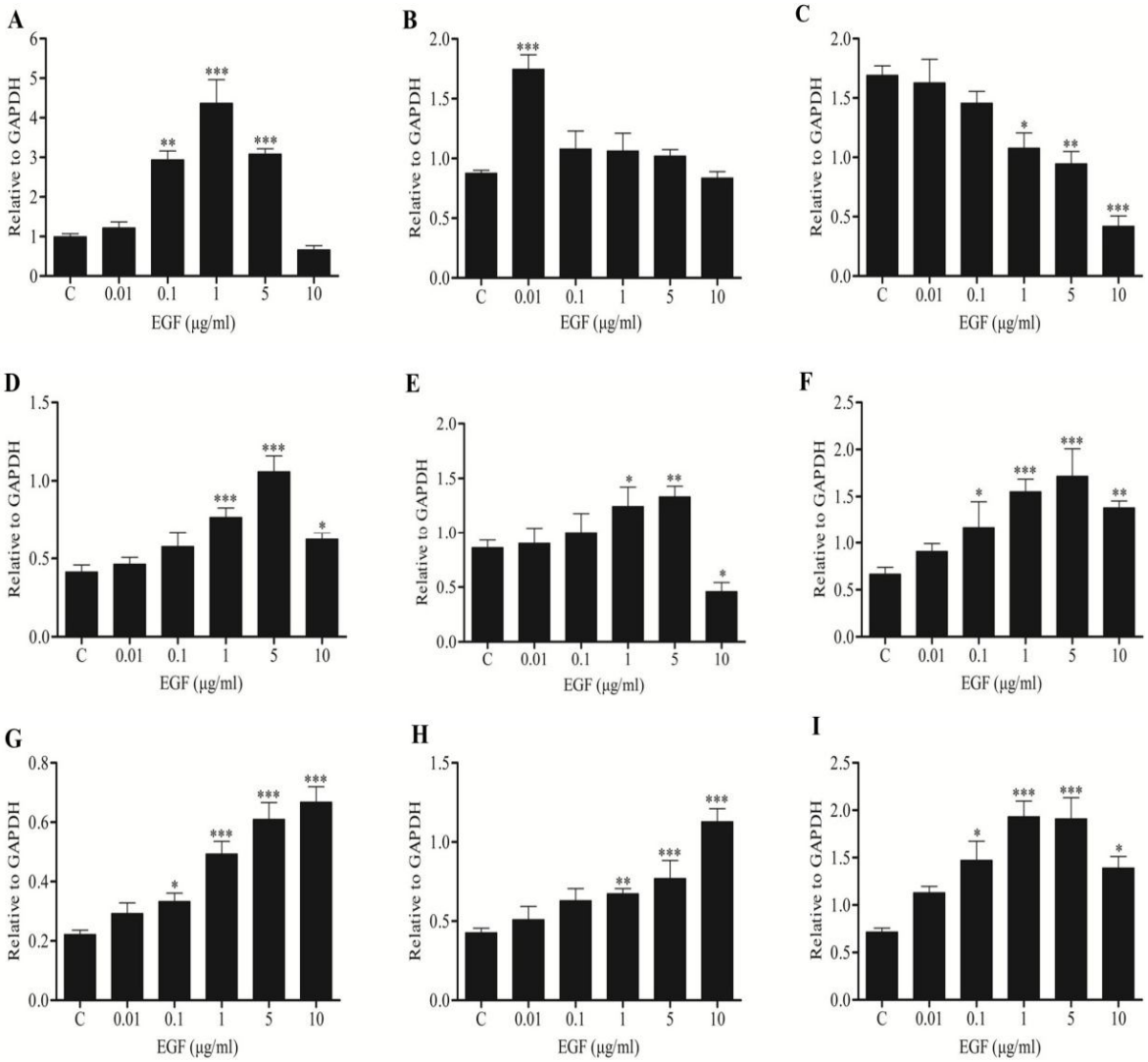


**Fig 3:** Effects of EGF on the expressions of three layers -specific markers. (A-C) The expression levels of AFP, ALB, and TTR in EBs under corresponding concentrations of EGF. (D-F) The expression levels of mesoderm-specific genes BMP4, GATA4 and MyoD. (G-I) The expression levels of ectoderm-specific genes GFAP and Pax6, and NF-H. Data presented are the mean O.D. values (±S.D.) of triplicate samples; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the control sample

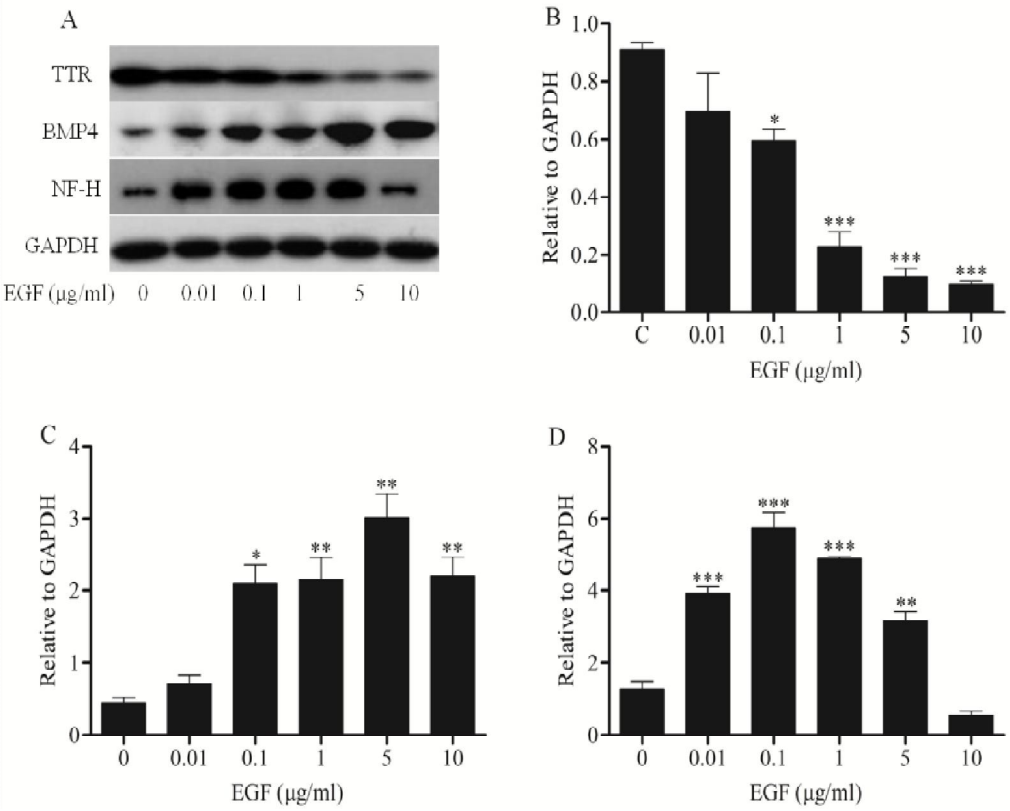


**Fig 4:** Effect of EGF on the expression of three layer-specific proteins. (A-D) The expression of TTR, BMP4, and NF-H in EBs under corresponding concentrations of EGF, were quantified with Western blot. Data are mean absorbance values (± SD) of triplicate measurements; versus control.

Alterations in the three proteins coincided with changes in the gene levels

## DISCUSSION

Developmental toxicity test systems have been established *in vivo* and *in vitro*. The in *vivo* tests are time-consuming and expensive, and are based on maternal or embryonic exposure of laboratory animals. Test models *in vitro* include those of whole embryo culture, ES cell micro mass cultivation, testicular cells, ovarian cells, and the EST [11]. With the advantage that only permanent cell lines are used, and consequently, no pregnant animals need be sacrificed to obtain embryonic cells, tissues or organs, EST validated by ECVAM was one of the most widely models to predict developmental toxicity [12]. ECVAM validation research has shown that prediction accuracy of EST for non- and weak embryotoxic compounds is 78%, and is 100% for strong embryotoxicants [13].

EGF is a small molecular protein that was first identified in mouse submandibular gland. Research has shown that EGF is a strong mitogen that effectively promotes and adjusts the growth and proliferation of skin cells, induces cells to secrete hyaluronic acid and glycoprotein, and uniquely protects the skin and mucous membrane [14]. Due to its extensive biological activities, EGF has been widely used in the clinic, as well as in cosmetic materials used in cosmetic surgery. For example, EGF promotes fetal lung maturity, and has been used in clinical trials for the prevention and treatment of neonatal respiratory distress syndrome [15]. In addition, research has shown that EGF is an autocrine factor that regulates early placental growth, development, and hormone synthesis function, and also participates in the regulatory function of first trimester villi gonadotropin secretion. It has been reported that the absence of EGF, following removal of the mouse submandibular gland, obviously increases the rate of fetal rat abortion and results in IUGR in rat fetuses [5], whereas the addition of EGF, improves the rate of embryo implantation and promotes nuclear and cytoplasmic maturation promoted by facilitation of the increase of gonadotrophins [16]. In contrast, Buyalos indicated that a low concentration of EGF promoted embryonic development, while a high concentration of EGF inhibited the development of the embryo [17]. However, as an extraneous drug, it is worth investigating whether EGF results in any adverse

impacts when used in the clinic.

In the present study, we first evaluated the embryotoxic potential of EGF using the classic EST protocol. The results revealed that EGF was a weak embryotoxicant, and so great attention should be paid to the fact that it may have embryotoxic potential when used in large doses, or long-term in low doses, especially in women in their first trimester of pregnancy.

Further, we investigated the way in which EGF made an impact on the expression of tissue-specific genes and proteins during the differentiation of mouse ES cells. The results showed that 0.01-10 μg/mL EGF had a suppressive differentiation effect, according to the expression levels of the undifferentiated genes Nanog and Oct4, which increased in a dose-dependent manner. As a whole, a relatively high concentration of EGF caused a discordant effect on the three germ layers. In particular, the expression of ectoderm-specific genes, including NF-H, GFAP, and Pax6, gradually increased at 0.01-5 μg/mL EGF concentrations. The expression levels of NF-H, an intermediate filament protein unique for neurons [18], reached a maximum at 1 μg/mL EGF, while it diminished slightly when the concentration of EGF increased. GFAP, a marker of radial glial cells, and Pax6, which play an important role in the eyes, and central nervous system tissue and organ formation, as well as in the process of embryonic development, showed sustained increasing expression [19], which is in accordance with a previous finding that EGF stimulates cells that originate from the ectoderm and endoderm, such as corneal, epithelial, breast, nerve, glial, and adrenal medulla cells, etc., resulting in their proliferation [20].

In the mesoderm lineage, the overall expression of BMP4 , GATA4, and MyoD showed a tendency to increase, and reached a maximum at 5 μg/mL. It is worth mentioning that at the mRNA and protein expression level of BMP4, both showed a significant increase, which inferred that EGF is important in accelerating bone growth and tooth development. Compared to the above, the expression of the ectoderm-specific gene AFP, which is widely used as a hepatic marker, showed a dose-dependent increase and decreased when the concentration of EGF exceeded 5 μg/mL, which suggests that a low concentration of EGF promotes embryonic stem cell differentiation into hepatic cells, while, toxicity at a high concentration of EGF leada to inhibition. It has been reported that 20-45 ng/mL EGF can be used as an inducer to promote hepatic cell directional differentiation from stem cells [21]. Our results showed the promotion of hepatic differentiation effects of EGF on increased expression of the AFP gene. Most notably, TTR, a marker of late differentiation stage, showed an obvious linear decrease, which may infer that TTR is sensitive to EGF. Combined with the analysis of protein, the changes of TTR affected by EGF were also verified. With regard to preliminary toxicity studies on other growth factors, aFGF and bFGF, both displayed particularly sensitive expression of gene TTR, so it can be preliminarily determined that TTR is a sensitive gene to those growth factors, and can be considered as a toxicity marker gene in our next study.

## CONCLUSION

Our results confirmed that EGF is a weak embryotoxicant. It could up-regulate the expressions of ectodermal- and mesodermal-specific genes, while it down-regulates or mostly has no impact on the expression of endoderm-specific genes at the same EGF concentration. The imbalance of the three layer-specific genes and protein expressions caused by EGF showed that EGF promoted the differentiation of the ectoderm and mesoderm, and inhibited or had mostly no impact on the endoderm at high concentrations. The sensitivity of TTR means that further investigation is required to determine whether TTR changes could be used as embryotoxicity biomarkers for growth factors.

## ACKNOWLEDGMENT

The study was supported by the National Natural Science Foundation of China (Grant no. 30973602) and the Natural Science Foundation of Guangdong Province, China (Grant no. S2011010001898), and for this, all the authors here express their deep thanks. Fen Chen and Fenfang Cao contributed equally to this work and should be considered co-first authors.

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