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Non-human primate and rodent embryonic stem cells are differentially sensitive to embryotoxic compounds

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ABSTRACT

Many industrial chemicals and their respective by-products need to be comprehensively evaluated for toxicity using reliable and efficient assays. In terms of teratogenicity evaluations, the murine-based embryonic stem cell test (EST) offers a promising solution to screen for multiple tissue endpoints. However, use of a mouse model in the EST can yield only a limited understanding of human development, anatomy, and physiology. Non-human primate or human *in vitro* models have been suggested to be a pharmacologically and pathophysiologically desirable alternative to murine *in vitro* models. Here, we comparatively evaluated the sensitivity of embryonic stem cells (ESCs) of a non-human primate to skeletal teratogens with mouse ESCs hypothesizing that inclusion of non-human primate cells in *in vitro* tests would increase the reliability of safety predictions for humans.

First, osteogenic capacity was compared between ESCs from the mouse and a New World monkey, the common marmoset. Then, cells were treated with compounds that have been previously reported to induce bone teratogenicity. Calcification and MTT assays evaluated effects on osteogenesis and cell viability, respectively. Our data indicated that marmoset ESCs responded differently than mouse ESCs in such embryotoxicity screens with no obvious dependency on chemical or compound classes and thus suggest that embryotoxicity screening results could be affected by species-driven response variation. In addition, ESCs derived from rhesus monkey, an Old World monkey, and phylogenetically closer to humans than the marmoset, were observed to respond differently to test compounds than marmoset ESCs. Together these results indicate that there are significant differences in the responses of non-human primate and mouse ESC to embryotoxic agents.

1. Introduction

In the United States, one in 28 babies carries congenital anomalies [1]. Although 50% of the causes for such birth defects are unknown, some may be traced back to involuntary environmental chemical exposure. There are more

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than 80,000 cataloged chemicals in the United States that may be released into the environment and most of them are inappropriately tested for safety. This lack of information is particularly concerning for sensitive populations such as pregnant women and children as adequate safety guidelines cannot always be confidently recommended. Furthermore, given that the developing fetus is especially sensitive to maternal environmental conditions and also that exposure during key points of development can lead to unique effects lasting through multiple generations [2], the potential embryotoxicity and teratogenicity of industrial compounds is of particular concern.

With appropriate data, acceptable exposure levels and actual safety of such products can be established for individuals that are most vulnerable to chemical exposure. Therefore, toxicology programs have been designed to identify toxicities that may potentially be encountered in human embryos. Under the worldwide trend for revision of chemical legislation, it will be necessary to test a large number of chemicals in a short time, which can only be achieved with predictive *in vitro* assays.

A step in the direction of animal sacrifice free embryotoxicity screen was taken when the classic embryonic stem cell test (EST) was first described [3,4]. This assay relies on embryonic stem cells (ESCs) from the mouse and compares two important aspects of prenatal toxicity. First, the EST has revealed the differences in sensitivity of mouse embryonic stem cells (ESCs) to chemical entities compared to adult fibroblasts. Second, the test determines the ability of a chemical to inhibit the differentiation of the ESCs into a differentiated cell type of interest [5,6].

Among the many birth defects, the ones that affect musculoskeletal tissues account for 5% of all infant deaths. Thus, skeletal toxicity has become a high priority screening phenotype and is currently integrated into the animal screens that assess general prenatal developmental toxicity (TG414, OECD) [7–9]. Assessing the inhibition of osteogenic differentiation of the ESCs, the EST may also be exploited to serve as predictor for developmental osteotoxicity [6,10–14].

Despite the routine use of rodent models in research, the mouse model as used in the EST can only yield a limited understanding of human development, anatomy and physiology. Accordingly, human *in vitro* models are desirable from a pharmacological and pathophysiological standpoint. Indeed, ESCs from humans were established around the turn of the century [15]. However, due to ethical considerations, the legality of their use varies widely between countries. A solution comes with human induced pluripotent stem cells (hiPSCs), which are artificially created from somatic cells, and are therefore not ethically challenged, but it is yet unclear how their quality or differentiation potential measures up to bona fide hESCs. Therefore, to provide a legal and ethical alternative to countries, which have banned hESC research, we test here whether the sensitivity of non-human primate ESCs to a small set of classical and skeletal embryotoxic agents is similar to that of mouse ESCs in order to evaluate whether the inclusion of non-human primate cells into the EST would increase the reliability of safety predictions for human use.

2. Materials and methods

2.1. Murine ESC maintenance

Murine D3 embryonic stem cells (American Type Culture Collection, Rockville, MD, USA) were expanded in high glucose DMEM containing L-glutamine (Corning). Media additionally contained 15% batch-tested fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM 2-mercaptoethanol (all Invitrogen) and 1000 U LIF/ml (Millipore). Cells were routinely passaged every 2–3 days with 0.25% Trypsin-EDTA (Life Technologies).

2.2. Maintenance culture of non-human primate ESCs

Embryonic stem cells from the common marmoset (cjes001) were cultured in feeder-free conditions as described [16]. Rhesus ESCs (R366.4, WiCell Research Institute) were cultured on mouse embryonic fibroblast feeder layers as previously described [17,18].

2.3. Osteogenic differentiation of ESCs

Murine ESCs were induced to differentiate *via* aggregation into embryoid bodies *via* hanging drops at 750 cells/drop, in the presence of control differentiation medium (CDM, mouse ESC maintenance medium without LIF [19]. Differentiating cells were replated on day 5 as a single cell suspension at a concentration of 50,000 cells/cm^2 [20]. Differentiation of marmoset and rhesus ESCs was initiated from intact ESC colonies in non-adherent conditions as described [16,17]. In brief, undifferentiated colonies were trypsinized with TrypLE (Invitrogen) into clusters of 20–30 cells. Approximately 100 such clusters were seeded in CDM to one bacteriological grade dish (100 mm diameter). Following 5 days of incubation, cell clusters were transferred onto cell culture plates coated with 0.1% gelatin at an approximate density of 10 cell clusters/cm^2. On day 5 of differentiation, cells from all species received osteogenic differentiation medium containing the induction factors β-glycerophosphate (10 mM), ascorbic acid (25 μg/ml), and 1α,25-(OH)$_2$ vitamin D$_3$ (5×10^{-8} M) in CDM.

2.4. Test compounds

5-fluorouracil, *all-trans* retinoic acid, penicillin G (all Sigma) were selected as control test compounds as the teratogenic potential of each has been well established by previous *in vivo* and *in vitro* investigations [21]. Stock solutions were made in DMSO and diluted to test concentrations in respective cell culture media. Lithium chloride was obtained from Fluka and aluminum chloride was obtained from Sigma. Sodium chloride (Fisher Scientific), lithium acetate (Aldrich), sodium acetate (Sigma), and aluminum hydroxide (Sigma) were included as controls for lithium and aluminum activity. Untreated control cultures containing appropriate vehicle were also included. Osteogenic differentiation was considered valid if the control solvent yielded osteoblast differentiation levels