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Historical Perspective

HISTORICAL PERSPECTIVE

A Historical Perspective on the Use of Stem/Progenitor Cell-Based *In Vitro* Methods for Neurodevelopmental Toxicity Testing

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A BRIEF HISTORY OF STEM CELL METHODS

Pluripotent cells from the inner cell mass of the mouse blastocyst were detected as uncommitted embryonic stem cells (ESCs) in the 1970s. These cells were found to have potent proliferative and differentiative capacities, the latter allowing them to contribute to tissues derived from ectoderm, endoderm and mesoderm when transferred to host blastocyst stage embryos (Gardner and Lyon, 1971; Gardner and Rossant, 1979). Successful isolation of these ESC resulted in the generation of stem cell lineages (Evans and Kaufman, 1981; Martin, 1981) that proliferate indefinitely without differentiation *in vitro*. Medium supplementation with fetal bovine serum as well as feeder cells have been preventing differentiation and supporting their growth. Primary fibroblasts (Doetschman *et al.*, 1985), permanent STO cell lines (Evans and Kaufman, 1981; Martin, 1981) or buffalo rat liver (Smith and Hooper, 1987) were used in the last century, while they were superseded by defined mouse embryonic fibroblast feeders in more recent times (Schmidt, 2001).

After successful cultivation of mouse ESC, which was also driven by the need for transgenic mice generation for disease modeling (Gossler *et al.*, 1986), the next milestone in this area was the preparation and cultivation of primate ESC. Here, the team of James Thomson was a pioneer by deriving ESC from rhesus monkey and marmoset blastocysts (Thomson *et al.*, 1995, 1996). Only 2 years later, the same team for the first time obtained and cultivated human ESC from surplus blastocysts donated by couples undergoing treatment for infertility. These cells still maintained the developmental potential to form trophoblast and derivatives of all 3 embryonic germ layers after undifferentiated proliferation for 4–5 months *in vitro* (Thomson *et al.*, 1998). Only shortly after, feeder cell-free culture conditions were developed that facilitate stem cell culture and maintenance (Xu *et al.*, 2001). Such defined culturing methods are supported especially for stem cell use in clinical applications currently (Hovatta *et al.*, 2014). For a more detailed description of this early ESC history and the vision for their possible application in different medical fields at this time, the interested reader is referred to Pera *et al.* (2000) and Odorico *et al.* (2001).

Although stem cells opened up large fields for cellular and molecular biology, the work on human ESC derived from human fertilized eggs also initiated ethical debates, ie, how to perform human ESC research while maintaining respect for human life (Robertson, 2001; Trepagnier, 2000). Due to general differences in ethical, theological and philosophical points of view within the general population, consensus on this matter has not been reachable. Therefore, the noble price-winning discovery of somatic cell reprograming into induced pluripotent stem cells (iPSCs) moved the stem cell field into a new dimension (Lowry *et al.*, 2008; Takahashi *et al.*, 2007; Takahashi and Yamanaka, 2006; Yu *et al.*, 2007). With such cells, stem cell work became possible for different biomedical applications devoid of the ethical issues associated with ESCs.

Year	Milestones of stem cell methods	Reference
70's	Detection of uncommitted ESC from the inner cell mass of the mouse blastocyst	Gardner and Lyon 1971 Gardner and Rossant 1979
1981	Generation of mouse embryonic stem cell lineages	Evans and Kaufman 1981 Martin 1981
1995	Cultivation of Rhesus monkey ESC	Thomson et al., 1995
1998	Cultivation of human ESC	Thomson et al.,1998
2001	Feeder-cell free culture conditions developed	Xu et al., 2001
2006	Discovery of somatic cell reprogramming into iPSC	Takahashi and Yamanaka 2006

Year	Milestones of SC methods in toxicological research	Reference
1993	Proposal to use ESC for reproductive toxicology	Vogel 1993
1994	Use of ESC for mutagenicity testing	Sehlmeyer and Wobus 1994
1994	Use of ESC for embryotoxicity testing	Wobus et al., 1994 Scholz et al., 1999
2000	Use of ESC for fertility impairment testing	Vogel et al., 2000
2001	Use of ESC for cardiac toxicity testing	Bremer et al., 2001
2003	Use of ESC for neurotoxicity testing	Qu et al., 2003
2011	Proposal to use hiPSC for neurotoxicity testing	Kumar et al., 2011
2016	Use of hiPSC for neurotoxicity testing	Pei et al., 2016 Ryan et al., 2016

Figure 1. Brief timeline depicting milestones of stem cell method development and their application in toxicology.

Besides PSC models, cultivation of organ-specific, multipotent stem/progenitor cells from rodents and humans were established (reviewed by Weissman *et al.*, 2001). Such have restricted transdifferentiation potential, yet are primary cell material with organ-specific developmental capacities. The discovery of the existence of central nervous system stem cells, ie, neural stem cell and neural progenitor cells (NPCs), began with the demonstration that cells derived from rodent fetal brain or adult subventricular zone or dentate gyrus can grow *in vitro* in the presence of defined factors such as epidermal growth factor and basic fibroblast growth factor. These cells can grow as adherent monolayers or as self-associating, free-floating neurospheres (Davis and Temple, 1994; Ray *et al.*, 1993; Reynolds and Weiss, 1992; Richards *et al.*, 1992; Temple and Alvarez-Buylla, 1999; Temple and Davis, 1994), which have the ability to self-renew and give rise to neurons, astrocytes and oligodendrocytes (Palmer *et al.*, 1997). Also from human fetal brains growth factor-responding neurospheres were generated that differentiate into the 3 major effector cells of the brain (Carpenter *et al.*, 1999) leading to the proposal of the existence of also human brain stem cells (Uchida *et al.*, 2000).

APPEARANCE OF STEM CELL METHODS IN TOXICOLOGICAL RESEARCH

Although the major concern for use of stem cells was regenerative medicine, toxicology soon made use of these translational cell systems. The very first article proposing usage of mouse ESC for regulatory toxicity testing was published by Vogel (1993), 13 years after the routine culture of mouse ESC was established (Evans and Kaufman, 1981; Martin, 1981). He proposed using ESC for reproductive toxicity screening (Vogel, 1993) and was quickly followed by others suggesting ESC-based *in vitro* assays for studying mutagenicity (Sehlmeyer and Wobus, 1994), embryotoxicity (Scholz *et al.*, 1999; Wobus *et al.*, 1994), fertility impairment (Vogel *et al.*, 2000), and cardiac toxicity (Bremer *et al.*, 2001). During this time, the concept of using stem cells for application in toxicology was strongly promoted (Davila *et al.*, 2004). The authors here clearly point out the usefulness of human ESC for toxicity evaluation due to their unique unlimited proliferation ability, plasticity to generate a large variety of cell types, and a more readily available source of human cells compared with primary human cells. It took until 2003 for the first study using ESC for neurotoxicity studies to be published (Qu *et al.*, 2003). This work was based on the creation of neuronal-glial cocultures, which at that time had been established by a variety of groups with different protocols and ESC from diverse species including humans (reviewed by McDonald, 2001). Later, after establishment of neuronal lineage hiPSC differentiation into brain region-specific neuroprogenitors, the great potential of hiPSC for neurotoxicity evaluation including mechanistical studies and human predicitve risk assessment was pointed out (reviewed by Kumar *et al.*, 2012) and a few years later neurotoxicity testing in hiPSC started to become reality (Pei *et al.*, 2016; Ryan *et al.*, 2016) (Figure 1).

DEVELOPMENTAL NEUROTOXICITY TESTING USING STEM/PROGENITOR CELL-BASED METHODS

The ability of stem cells to differentiate into the neuroectodermal lineage opened the door for evaluating compounds that interfere with the development of the nervous system, i.e. studying developmental neurotoxicity (DNT). Since 2005, international researchers have been developing concepts how to use and interpret such alternative methods with the final goal of regulatory application (Bal-Price *et al.*, 2012, 2015, 2018a; Crofton *et al.*, 2011; Fritsche *et al.*, 2017, 2018; Lein *et al.*, 2005). Here, the concept evolved that the complex procedure of brain development is disassembled into spatiotemporal neurodevelopmental processes that are necessary for forming a brain. According to the adverse outcome pathway concept, such are key events for DNT that can be tested for adverse effects of compounds in *in vitro* assays (Bal-Price *et al.*, 2015, 2018a). Such key events include stem cell differentiation into NPCs, neural proliferation and apoptosis, NPC migration, neuronal and glia differentiation and maturation, neurite outgrowth, synaptogenesis and neuronal network formation. Recently, the need for stem cell-based *in vitro* testing for DNT evaluation was pointed out (Bal-Price *et al.*, 2015, 2018b; Fritsche *et al.*, 2017; Singh *et al.*, 2016). This need is currently taken up by putting a DNT testing battery into place that utilizes the most ready assays for compound screening (Bal-Price *et al.*, 2018a). In the future, DNT *in vitro* testing will be expanded as basic knowledge on brain development magnifies and stem/progenitor cell-based methods that are able to screen for example, astrocyte differentiation, maturation and function, neuronal maturation and network formation, brain region-specific traits as well as molecular aspects of hormone-related and/or sex-specific neural cell functions become available. These are endpoints that have not been fully developed into stem/progenitor cell-based test methods yet, but belong to the indispensable neurodevelopmental key events of a powerful and predictive DNT testing battery.

REFERENCES

Bal-Price, A., Crofton, K. M., Leist, M., Allen, S., Arand, M., Buetler, T., Delrue, N., FitzGerald, R. E., Hartung, T., Heinonen, T., *et al.* (2015). International STakeholder NETwork (ISTNET): Creating a developmental neurotoxicity (DNT) testing road map for regulatory purposes. *Arch Toxicol* **89**, 269–287.

Bal-Price, A., Hogberg, H. T., Crofton, K. M., Daneshian, M., FitzGerald, R. E., Fritsche, E., Heinonen, T., Hougaard Bennekou, S., Klima, S., Piersma, A. H., *et al.* (2018a). Recommendation on test readiness criteria for new approach methods in toxicology: Exemplified for developmental neurotoxicity. *ALTEX*: 1709221.

Bal-Price, A., Pistollato, F., Sachana, M., Bopp, S. K., Munn, S., and Worth, A. (2018b). Strategies to improve the regulatory assessment of developmental neurotoxicity (DNT) using in vitro methods. *Toxicol. Appl. Pharmacol.*

Bal-Price, A. K., Coecke, S., Costa, L., Crofton, K. M., Fritsche, E., Goldberg, A., Grandjean, P., Lein, P. J., Li, A., Lucchini, R., *et al.* (2012). Advancing the science of developmental neurotoxicity (DNT): Testing for better safety evaluation. *Altex Altern. Anim. Exp.* **29**, 202–215.

Bremer, S., Worth, A. P., Paparella, M., Bigot, K., Kolossov, E., Fleischmann, B. K., Hescheler, J., and Balls, M. (2001). Establishment of an in vitro reporter gene assay for developmental cardiac toxicity. *Toxicol. In Vitro* **15**, 215–223.

Carpenter, M. K., Cui, X., Hu, Z. Y., Jackson, J., Sherman, S., Seiger, A., and Wahlberg, L. U. (1999). In vitro expansion of a multipotent population of human neural progenitor cells. *Exp. Neurol.* **158**, 265–278.

Crofton, K. M., Mundy, W. R., Lein, P. J., Bal-Price, A., Coecke, S., Seiler, A. E., Knaut, H., Buzanska, L., and Goldberg, A. (2011). Developmental neurotoxicity testing: Recommendations for developing alternative methods for the screening and prioritization of chemicals. *ALTEX* **28**, 9–15.

Davila, J. C., Cezar, G. G., Thiede, M., Strom, S., Miki, T., and Trosko, J. (2004). Use and application of stem cells in toxicology. *Toxicol. Sci.* **79**, 214–223.

Davis, A. A., and Temple, S. (1994). A self-renewing multipotential stem cell in embryonic rat cerebral cortex. *Nature* **372**, 263–266.

Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* **87**, 27–45.

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156.

Fritsche, E., Crofton, K. M., Hernandez, A. F., Bennekou, S. H., Leist, M., Bal-Price, A., Reaves, E., Wilks, M. F., Terron, A., Solecki, R., *et al.* (2018). Consensus statement on the need for innovation, transition and implementation of developmental neurotoxicity (DNT) testing for regulatory purposes. *Toxicol. Appl. Pharmacol.*

Fritsche, E., Crofton, K. M., Hernandez, A. F., Bennekou, S. H., Leist, M., Bal-Price, A., Reaves, E., Wilks, M. F., Terron, A., Solecki, R., *et al.* (2017). OECD/EFSA workshop on developmental neurotoxicity (DNT): The use of non-animal test methods for regulatory purposes. *Altex Altern. Anim. Exp.* **34**, 311–315.

Gardner, R. L., and Lyon, M. F. (1971). X chromosome inactivation studied by injection of a single cell into the mouse blastocyst. *Nature* **231**, 385–386.

Gardner, R. L., and Rossant, J. (1979). Investigation of the fate of 4-5 day post-coitum mouse inner cell mass cells by blastocyst injection. *J. Embryol. Exp. Morphol.* **52**, 141–152.

Gossler, A., Doetschman, T., Korn, R., Serflingt, E., and Kemler, R. (1986). Transgenesis by means of blastocyst-derived embryonic stem cell lines. *Dev. Biol.* **83**, 9065–9069.

Hovatta, O., Rodin, S., Antonsson, L., and Tryggvason, K. (2014). Concise review: Animal substance-free human embryonic stem cells aiming at clinical applications. *Stem Cells Transl. Med.* **3**, 1269–1274.

Kumar, K. K., Aboud, A. A., and Bowman, A. B. (2012). The potential of induced pluripotent stem cells as a translational model for neurotoxicological risk. *Neurotoxicology* **33**, 518–529.

Lein, P., Silbergeld, E., Locke, P., and Goldberg, A. M. (2005). In vitro and other alternative approaches to developmental neurotoxicity testing (DNT). *Environ. Toxicol. Pharmacol.* **19**, 735–744.

Lowry, W. E., Richter, L., Yachechko, R., Pyle, A. D., Tchieu, J., Sridharan, R., Clark, A. T., and Plath, K. (2008). Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 2883–2888.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **78**, 7634–7638.

McDonald, J. W. (2001). ES cells and neurogenesis. In *Stem Cells and CNS Development* (M. S. Rao, Ed.), pp. 207–261. Humana Press Inc., Totowa, NJ.

Odorico, J. S., Kaufman, D. S., and Thomson, J. A. (2001). Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* **19**, 193–204.

Palmer, T. D., Takahashi, J., and Gage, F. H. (1997). The adult rat hippocampus contains primordial neural stem cells. *Mol. Cell Neurosci.* **8**, 389–404.

Pei, Y., Peng, J., Behl, M., Sipes, N. S., Shockley, K. R., Rao, M. S., Tice, R. R., and Zeng, X. (2016). Comparative neurotoxicity screening in human iPSC-derived neural stem cells, neurons and astrocytes. *Brain Res.*

Pera, M. F., Reubinoff, B., and Trounson, A. (2000). Human embryonic stem cells. *J. Cell Sci.* **113(Pt 1)**, 5–10.

Qu, Y., Vadivelu, S., Choi, L., Liu, S., Lu, A., Lewis, B., Girgis, R., Lee, C. S., Snider, B. J., Gottlieb, D. I., *et al.* (2003). Neurons derived from embryonic stem (ES) cells resemble normal neurons in their vulnerability to excitotoxic death. *Exp. Neurol.* **184**, 326–336.

Ray, J., Peterson, D. A., Schinstine, M., and Gage, F. H. (1993). Proliferation, differentiation, and long-term culture of primary hippocampal neurons. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 3602–3606.

Reynolds, B. A., and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707–1710.

Richards, L. J., Kilpatrick, T. J., and Bartlett, P. F. (1992). De novo generation of neuronal cells from the adult mouse brain. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 8591–8595.

Robertson, J. A. (2001). Human embryonic stem cell research: Ethical and legal issues. *Nat. Rev. Genet.* **2**, 74–78.

Ryan, K. R., Sirenko, O., Parham, F., Hsieh, J.-H., Cromwell, E. F., Tice, R. R., and Behl, M. (2016). Neurite outgrowth in human induced pluripotent stem cell-derived neurons as a high-throughput screen for developmental neurotoxicity or neurotoxicity. *Neurotoxicology* **53**, 271–281.

Schmidt, J. V. (2001). Embryonic stem (ES) cell culture basics. *Curr. Protoc. Toxicol.* Chapter 15, Unit15 1.

Scholz, G., Pohl, I., Genschow, E., Klemm, M., and Spielmann, H. (1999). Embryotoxicity screening using embryonic stem cells in vitro: Correlation to in vivo teratogenicity. *Cells Tissues Organs* **165**, 203–211.

Sehlmeyer, U., and Wobus, A. M. (1994). Lower mutation frequencies are induced by ENU in undifferentiated embryonic cells than in differentiated cells of the mouse in vitro. *Mutat. Res.* **324**, 69–76.

Singh, S., Srivastava, A., Kumar, V., Pandey, A., Kumar, D., Rajpurohit, C. S., Khanna, V. K., Yadav, S., and Pant, A. B. (2016). Stem cells in neurotoxicology/developmental neurotoxicology: Current scenario and future prospects. *Mol. Neurobiol.* **53**, 6938–6949.

Smith, A. G., and Hooper, M. L. (1987). Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. *Dev. Biol.* **121**, 1–9.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676.

Temple, S., and Alvarez-Buylla, A. (1999). Stem cells in the adult mammalian central nervous system. *Curr. Opin. Neurobiol.* **9**, 135–141.

Temple, S., and Davis, A. A. (1994). Isolated rat cortical progenitor cells are maintained in division in vitro by membrane-associated factors. *Development* **120**, 999–1008.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147.

Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P., Becker, R. A., and Hearn, J. P. (1995). Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7844–7848.

Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P., and Hearn, J. P. (1996). Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. *Biol. Reprod.* **55**, 254–259.

Trepagnier, D. M. (2000). Human embryonic stem cell research: Implications from an ethical and legal standpoint. *J. La State Med. Soc.* **152**, 616–624.

Uchida, N., Buck, D. W., He, D., Reitsma, M. J., Masek, M., Phan, T. V., Tsukamoto, A. S., Gage, F. H., and Weissman, I. L. (2000). Direct isolation of human central nervous system stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 14720–14725.

Vogel, R. (1993). In vitro approach to fertility research: Genotoxicity tests on primordial germ cells and embryonic stem cells. *Reprod. Toxicol.* **7**, 69–73.

Vogel, R., Domke, A., Tenschert, B., and Bremer, S. (2000). In vitro germ cell models for the detection of fertility impairment. *Andrologia* **32**, 219–224.

Weissman, I. L., Anderson, D. J., and Gage, F. (2001). Stem and progenitor cells: Origins, phenotypes, lineage commitments, and transdifferentiations. *Annu. Rev. Cell Dev. Biol.* **17**, 387–403.

Wobus, A. M., Rohwedel, J, Maltsev, V., and Hescheler, J. (1994). In vitro differentiation of embryonic stem cells into cardiomyocytes or skeletal muscle cells is specifically modulated by retinoic acid. *Roux Arch. Dev. Biol.* **204**, 36–45.

Xu, C., Inokuma, M. S., Denham, J., Golds, K., Kundu, P., Gold, J. D., and Carpenter, M. K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nat. Biotechnol.* **19**, 971–974.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., *et al.* (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920.