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Review

The contribution of human/non-human animal chimeras to stem cell research

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ABSTRACT

Chimeric animals are made up of cells from two separate zygotes. Human/non-human animal chimeras have been used for a number of research purposes, including human disease modeling. Pluripotent stem cell (PSC) research has relied upon the chimera approach to examine the developmental potential of stem cells, to determine the efficacy of cell replacement therapies, and to establish a means of producing human organs. Based on ethical issues, this work has faced pushback from various sources including funding agencies. We discuss here the essential role these studies have played, from gaining a better understanding of human biology to providing a stepping stone to human disease treatments. We also consider the major ethical issues, as well as the current status of support for this work in the United States.

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1. Introduction

A chimera is an organism that has more than a single set of parents, and consists of cells from two zygotes. They can be intraspecific, for example if two early stage embryos of the same species fuse to make a single embryo, or interspecific, as with human stem cell/non-human animal chimeras discussed here. The classic chimera from Greek Mythology is part lion, part goat, part snake. Chimera-based studies using human cells combined with a non-human species have been a critical line of investigation for basic and applied research in a number of areas of biology, including cancer and immunology (Behringer, 2007). Pluripotent stem cell (PSC) chimeras provide a unique platform for asking basic questions about embryonic development, for testing the efficacy of cell replacement therapies in animal models, and for building human organs (Wu et al., 2016).

Embryonic stem cells (ESCs) can become virtually any cell in the body. Since the derivation of the first human ESC lines, there has been great excitement surrounding the prospect of using these cells to treat human disease or injury (Trounson and McDonald, 2015; Ilic and

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Ogilvie, 2017). The excitement grew with the generation of induced pluripotent stem cells (iPSC), derived from adult somatic tissue, such as skin cells, through forced expression of a handful of key genes (Takahashi and Yamanaka, 2006). iPSCs share many properties with ESCs, including their broad differentiation capacity. In theory, they can be derived from a specific patient who could benefit from a cell transplantation therapy, avoiding the problem of genetic incompatibility. iPSCs also sidestep the controversial ethical issues surrounding the human embryo origin of ESCs (Gruen et al., 2007; Hyun, 2013). Although a number of roadblocks restrict their clinical use at present, including concerns that the genetic modifications used to generate most iPSC lines may lead to subsequent tumor formation when the cells are used for cell-based therapies, these issues will likely be resolved in the near future (Shi et al., 2017).

Where do we currently stand with clinical applications using PSCs, either ESCs or iPSCs? A number of clinical trials using these cells are underway in the United States, with additional disease targets in the planning stages (Trounson and McDonald, 2015). In each of these instances, prior to FDA approval for a clinical trial, it was essential to establish efficacy of the approach by providing extensive pre-clinical data using human PSC derivatives transplanted into a relevant rodent disease or injury model. There is a great demand for a new source of human organs. Progress is being made towards using human PSCs to build human organs in other species, likely pigs (Bourret et al., 2016; Wu and Izpisua Belmonte, 2016). This approach may involve combining human PSCs with early embryos of a host species using an approach called blastocyst complementation (Wu and Izpisua Belmonte, 2016). Both of these research directions focus on treating human disease and injury, and both require the formation of human-animal chimeras, either for pre-clinical testing or for eventual production of donor organs. Human PSC/non-human animal chimeras can also be used to model disease and provide a platform for drug testing in an *in vivo* environment that more closely mimics the human condition than can be achieved with cell culture studies.

But controversy, based primarily upon ethical concerns, surrounds the use of human/non-human animal chimeras and may impede future progress (Streiffer, 2010; Hermeren, 2015; Hyun, 2015). In this review, we examine the use of PSC human/non-human animal chimeras. What have we learned about development in general, and human development in particular? How have chimera studies facilitated a move towards translation? We will also consider the relevant ethical issues and the move towards establishing guidelines for oversight of this work.

2. PSC chimeras as tools to study development

Since many differences between the developmental programs of human and mouse embryos have been identified, it is essential to study human development directly, and not simply extrapolate from the well-studied mouse model. Distinctions between the species include the morphology of the epiblast, formation of extraembryonic lineages, and speed of differentiation (Rossant, 2015). There are, however, many challenges to studying human development directly. Ethical issues surrounding the moral status of the human embryo have led to restrictions, varying from country to country, on working with human embryos. Since 1996, in the United States, the Dickey-Wicker amendment, a rider Congress places on the appropriations bill each year, prohibits the use of federal funds for research that involves manipulating or destroying human embryos (Rodriguez et al., 2011). Such studies remain legal however, and can proceed with alternative funding sources such as private corporations or foundations. In the United Kingdom, all research involving human embryos must be approved by the Human Fertilisation and Embryo Authority, whether originating from the public or private sector. Consensus from regulatory guidelines limits the *in vitro* culture of human embryos to the gastrulation stage, around 14 days post-fertilization, though there have been recent calls to re-evaluate this time limitation (Hyun et al., 2016).

Examining the fate of human PSCs in interspecific chimeras is an alternative approach for studying human development that also provides information on the pluripotency of the human cell lines used. The work of Le Douarin set the stage for interspecific chimera work, using chick-quail chimeras to follow cell lineage (Le Douarin, 1980). Mouse/rat chimeras were used for lineage analysis of mammalian embryos (Gardner and Johnson, 1973), and sheep/goat chimeras to investigate reproductive barriers (Fehilly et al., 1984). Interspecific chimeras have the best outcomes, with substantive contribution from the test cells, when the species are close evolutionary matches (Wu et al., 2016). Discrepancies in the size of the embryos, stage of host and donor tissue, use of signaling pathways, or gestation time can provide serious constraints on successful integration of human cells into host embryos (Wu et al., 2016; Wu and Izpisua Belmonte, 2016). These concerns explain the minimal success observed when adding human PSCs to the embryos of xenogenic hosts.

Initial attempts to form human-mouse chimeras using injection of human PSCs into mouse morula or blastocysts showed minimal, transient chimerism (James et al., 2006; Wu et al., 2015). When it was revealed that human ESCs represent a “primed” pluripotent state similar to the post-implantation epiblast stage rather than the “naïve” inner cell mass-like state observed for mouse ESCs (Tesar et al., 2007; Savatier et al., 2017), the mismatch in developmental stage - epiblast-like test cells and a blastocyst host - provided a likely explanation for the poor success observed. Chimeras made with human PSCs and epiblast stage mouse embryos result in more substantial integration of the human cells (Wu et al., 2015; Mascetti and Pedersen, 2016). These studies used *ex-vivo* culture of the chimeras and revealed incorporation of the human PSCs into derivatives of all 3 primary germ layers, based upon marker expression, although chimerism was generally not robust. Recently many protocols for producing naïve human PSCs have been described, with a naïve *versus* primed state defined by a number of parameters, including growth factor requirements, PSC morphology, gene expression profile, and epigenetic state. The first report generating ERK-independent naïve human PSCs demonstrated their integration at the mouse blastocyst stage and subsequent contribution to host tissues at 12.5 days (Gafni et al., 2013). Although additional studies reveal some variation in the reproducibility and extent of chimerism with mouse embryos observed using these cell lines, all reports are consistent with integration of the naïve hPSCs at the blastocyst stage and some document contribution to tissues at post-implantation stages (Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Theunissen et al., 2016). Variation in the frequency and extent of chimerism reported for the naïve cell lines could be attributed to the manner in which the cell line was generated, leading to discrepancies in epigenetic landscape, or alternatively to the sensitivity of assays employed to monitor the fate of the human cells.

Given the need for a close match for embryo size and evolutionary distance, researchers have begun to use large animal hosts to examine human PSC fate. In a recent report, cattle or pig blastocyst hosts supported the incorporation of naïve, intermediate, or primed human PSCs, though limited incorporation was observed with the more mature primed cells (Wu et al., 2017). The experiments were taken further in the pig hosts and chimeric embryos were transferred to maternal surrogates and the extent of chimerism evaluated at post-implantation stages (Wu et al., 2017). The human iPS cells contributed to several tissues reflecting all three germ layers. However, chimerism was limited and the presence of PSCs frequently led to abnormal embryonic development. More efficient chimera formation has been observed using more lineage-restricted PSC-derived cell types added to later stage embryos (Jaenisch, 1985), neonates, or adults, and this approach will be discussed under the *PSC chimeras to test the efficacy of a cell-based therapy* section.

Although the focus of this review is on human/non-human animal chimera research, it is worth noting advances in the field using non-human primate PSCs. Cynomolgus monkey naïve ESCs, generated