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SPOTLIGHT

Interspecies chimeras for human stem cell research

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ABSTRACT

Interspecies chimeric assays are a valuable tool for investigating the potential of human stem and progenitor cells, as well as their differentiated progeny. This Spotlight article discusses the different factors that affect interspecies chimera generation, such as evolutionary distance, developmental timing, and apoptosis of the transplanted cells, and suggests some possible strategies to address them. A refined approach to generating interspecies chimeras could contribute not only to a better understanding of cellular potential, but also to understanding the nature of xenogeneic barriers and mechanisms of heterochronicity, to modeling human development, and to the creation of human transplantable organs.

KEY WORDS: Pluripotent stem cell, Interspecies chimera, Heterochronicity, Chimeric assays, Human development

Introduction

The generation of chimeras has been a classic and common approach in developmental biology and is used to analyze developmental potency by transplanting cells into a genetically different host. Although there are other approaches to assess cellular dynamics, such as genetic lineage tracing, the importance of chimeric assays has increased recently alongside advances in the field of stem cell biology. Whatever pluripotent stem cells (PSCs) we isolate or generate, we usually predict their differentiation potential by gene expression profile or by *in vitro* functional assays. However, a definitive conclusion cannot be made until their developmental potency is confirmed *in vivo* by making a chimera. The ability to contribute to chimera formation across multiple lineages when injected into the pre-implantation blastocyst is still a gold standard for 'true' PSCs, at least when both donor and host cells belong to the same species. This is a convenient and feasible method if one wants to determine the differentiation potential of stem cells derived from laboratory animals such as mice. However, when it comes to human PSCs (), chimeric assays become extremely difficult. For these cells, given the ethical concerns, a xenogeneic setting – meaning cross-species chimerism – is the only possible choice for using a chimeric assay. It is worth noting, however, that unlike chimera formation between syngeneic or allogeneic species, there are a number of factors that can differ significantly between species in the xenogeneic setting. These include the structure of ligands and/or adhesive molecules, the developmental system itself, cell proliferation rate and so on. Given these differences, it is important to remember that a failure to generate full interspecies chimeras using human PSCs does not necessarily mean that the cells are not pluripotent, but possibly that a suitable host species and environment have not yet been identified. As such, it is best to employ a chimeric assay between the same or closely related species.

Guidelines from the International Society for Stem Cell Research (ISSCR) and the relevant regulatory authorities of many countries indicate that there are ethical concerns regarding chimeric experiments performed using human or non-human primate embryos, even before the pre-implantation stage. For example, ISSCR guidelines (as of 2016) allow for the development of human embryos injected with human PSCs only *in vitro* and only up to gastrulation. *In vitro* culture of mouse embryos injected with human PSCs might demonstrate whether these cells have ability to contribute to mouse epiblast (Masaki et al., 2015); however, further analysis of their differentiation potential is impossible in such a short period of time. Therefore, in order to uncover the actual potency of human stem cells *in vivo*, an interspecies chimeric assay becomes crucial. This is particularly true for human naïve-like PSC lines (Theunissen et al., 2016), since there are a variety of conditions under which they can be established (Li et al., 2009; Hanna et al., 2010; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014). In addition, it is becoming increasingly important to verify the *in vivo* functionality of cells derived *in vitro* from PSCs, or indeed other stem cell types. In this Spotlight article, we provide some examples of how interspecies chimeric assays can and should be applied to determine the developmental potency of human PSCs, somatic stem cells, or cells derived *in vitro* from stem cells. We further discuss some important considerations for performing chimeric assays, such as the evolutionary distance between species, the nature of the cells themselves and some of the ethical concerns that have arisen in the field.

Human PSC-animal chimeras and the importance of developmental timing

The pluripotent nature of human embryonic stem cells (hESCs) has been demonstrated *in vitro* and by teratoma formation *in vivo* (Thomson et al., 1998). Goldstein et al. (2002) demonstrated that the transplantation of hESCs into somite stage chick embryos resulted in the autonomous formation of neural tube-like structure by hESC-derived cells. In mammals, James et al. (2006) transplanted hESCs into mouse blastocysts, developed in uterus, and showed that hESC-derived cells survived only in malformed embryos and were absent from normally developed embryos. Based on the gene expression profile, X-chromosome inactivation state and other unique characteristics, non-rodent PSCs, including those from human, are considered to be more like epiblast stem cells (EpiSCs), a type of PSC akin to the post-implantation stage of rodents (Brons et al., 2007; Tesar et al., 2007), than pre-implantation stage, naïve PSCs. When EpiSCs were transplanted into pre-implantation embryos, they were unable to form chimeras, unlike rodent ESCs (Brons et al., 2007; Tesar et al., 2007). Consistent with this, chimera formation was also not possible when monkey ESCs were transplanted into monkey embryos (Tachibana et al., 2012). On the other hand, it is

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possible to generate chimeras by transplanting EpiSCs into egg-cylinder stage embryos, which is the developmental stage when the epiblast is obtained to establish EpiSCs (Huang et al., 2012; Kojima et al., 2014). Consistent with these findings, hESCs transplanted into mouse egg-cylinder embryos survive and are able to differentiate into multiple lineages (Wu et al., 2015; Mascetti and Pedersen, 2016). These reports suggested that synchronization of developmental stage between the transplanted PSCs and the host embryo is required for chimera formation. Therefore, the formation of chimeras when naïve PSCs are injected into the pre-implantation embryo should prove that the transplanted cells are truly naïve.

Evolutionary distance is a barrier to chimera formation

Some human naïve-like PSCs have been reported to form interspecies chimeras when injected into mouse pre-implantation embryos (Gafni et al., 2013; Theunissen et al., 2016). However, in both cases, the frequency of chimeric embryos among all injected embryos was remarkably low, and the degree of chimerism – the contribution of the PSC-derived cells to the chimera – was far below the level found in mouse-rat interspecies chimera (Kobayashi et al., 2010; Isotani et al., 2011; Yamaguchi et al., 2017; Wu et al., 2017). Other groups have reported that engrafted human naïve-like PSCs disappear after implantation, or in some cases survive but contribute only to the extraembryonic region (Takashima et al., 2014; Masaki et al., 2015). Rodents are genetically more distant from humans than are other animals such as pigs or monkeys and early events in rodent embryogenesis are also unique. Therefore, results reported using mouse embryos might differ from those obtained if the human cells were to be transplanted into embryos of more closely related animals. Indeed, interspecies chimeras have been successfully generated between closely related animals, such as sheep-goat and mouse-rat (Fehilly et al., 1984; Kobayashi et al., 2010). So, although mouse is the most accessible animal in terms of obtaining embryos, when analyzing human cells other hosts more closely related to humans should be considered. In support of this, Wu et al. (2017) reported successful chimera formation using human primed PSCs transplanted into porcine embryos – albeit at a very low rate and degree of chimerism – whereas chimeras were not obtained when rodent naïve PSCs were transplanted. These results highlight the importance of genetic/evolutionary distance when generating interspecies chimeras. Interestingly, Yang et al. (2017) recently reported that newly established human PSCs with an expanded developmental potential showed an improved rate of chimerism with mouse embryos. However, whether the cells might show an even higher rate of chimerism with porcine embryos was not tested. It has also been shown that human PSC-derived cells in interspecies chimeras can differentiate into multiple lineages (Mascetti and Pedersen, 2016; Wu et al., 2015, 2017; Yang et al., 2017), but whether their differentiation was autonomous or due to cooperative morphogenesis with host tissue is controversial. It remains to be seen whether organs and tissues can be cooperatively formed between host cells and transplanted human cells in these interspecies chimeras.

Chimera generation by apoptosis-resistant cells

When mouse EpiSCs are transplanted into a mouse pre-implantation embryo, they do not form a chimera (Brons et al., 2007; Tesar et al., 2007). In a recent study, Masaki et al. (2015) closely observed the fate of mouse EpiSCs as well as mouse ESCs after injection into blastocysts by live imaging. Surprisingly, all of the injected EpiSCs were dead within 24 h post-injection, whereas most of the ESCs survived (Masaki et al., 2015). The authors hypothesized that the transplanted cells underwent apoptosis, and that by inhibiting apoptosis EpiSCs might be able to contribute to chimera formation. Indeed, forced expression of the apoptosis regulator BCL2 in mouse and rat EpiSCs allowed these cells to form chimeras when introduced into pre-implantation mouse blastocysts. Furthermore, not only EpiSCs, but also apoptosis-resistant endoderm progenitor cells, could form region-specific chimeras after transplantation into pre-implantation blastocysts (Masaki et al., 2016). If this system can also be applied to human cells, then it might prove possible to assess the developmental potency of cells by interspecies chimera assay.

The data from the Masaki et al. (2016) study are intriguing for a number of reasons. First, they seem to hint at the existence of a mechanism that eliminates cells that are unmatched in terms of developmental stage by apoptosis. This might have implications for characterizing naïve versus primed PSCs, as one of the key differences between these cells may be the ability to survive in the pre-implantation blastocyst environment. It was also surprising that the apoptosis-resistant endodermal progenitor cells survived and maintained their developmental cell fate. It seemed as if the cells were able to pause their fate for several days in stage-unmatched embryos, before resuming differentiation alongside the host cells when the appropriate developmental stage arose. How the cells were able to achieve such a ‘pause and replay’ in stage-unmatched embryos is an extremely interesting question.

Human somatic cell-derived chimeras with post-implantation animals

There are many examples of interspecies chimeras whereby human cells other than PSCs are transplanted. Animals with human blood can be prepared by transplanting human hematopoietic stem progenitor cells (HSPCs) into severely immunodeficient animals such as NOD/SCID mice and NSG mice (Shultz et al., 1995; Larochelle et al., 1996; Traggiai et al., 2004). These mice have been used extensively to analyze the physiological functions of human HSPCs. Interspecies hematopoietic chimeras have also been generated in large animals, such as sheep or pigs, by *in utero* transplantation of human HSPCs into embryos, taking advantage of the immune tolerance afforded by these animals (Zanjani et al., 1992; Fujiki et al., 2003). In this system, the contribution of human cells to the peripheral blood was generally very low, and thus it might be worth considering strategies to ‘empty’ the host hematopoietic stem cell (HSC) niche prior to transplantation, as others have shown in the field of interspecies organ generation (Kobayashi et al., 2010). Suzuki et al. (2013) showed that human HSCs can be produced from human induced pluripotent stem cells (iPSCs) by *in vivo* teratoma formation in mouse. Since the production of bona fide HSCs from human iPSCs has not yet been achieved *in vitro*, these data are interesting as they might suggest that using the *in vivo* biological environment could provide an advantage over *in vitro* culture.

For the purposes of analyzing the physiological function and potential of human neuronal cells, interspecies chimeric assays using cells of the neural lineage – either neural stem cells (NSCs) or their more differentiated progeny – have been performed. For example, human glial progenitor cells transplanted into the mouse brain are able to differentiate, mature and form a neural network with the neurons of the host animal (Han et al., 2013). Other progenitor cell types, for example neural crest cells, have also been used in interspecies chimeric assays (Cohen et al., 2016). In this case, the neural crest cells were derived *in vitro* from human PSCs and transplanted into the mouse embryos at the appropriate location

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and stage of development. The transplanted cells differentiated into melanocytes *in vivo* and contributed to coat color chimerism. Unlike PSCs, these committed stem/progenitor cells may engraft better in xenogeneic, non-synchronized environments by bypassing early, highly regulated developmental processes.

Ethical concerns and future perspectives

A concern in the context of human-animal chimera generation is the possibility of producing human gametes. If spermatozoa or eggs were to be formed in interspecies chimera, and if these chimeras were to cross with each other, there is the possibility that a human conceptus may be created. Although it is generally accepted that a human embryo could not develop further than the implantation stage in the uterus of another animal because of physiological and anatomical differences, even the temporary creation of a human embryo would raise significant ethical and moral issues. An additional concern with the generation of interspecies chimeras is that it is difficult to define the boundary between what is human and what is animal. No one knows what level of chimerism would be needed to attain human-like high-level brain functions or to generate human gametes. Thus, it will be important to carefully characterize the degree of chimerism by human cells in gametes and the central nervous system at the immature fetal stage. Judging from the results published so far for human-animal interspecies chimeras (Masaki et al., 2015; Wu et al., 2017), it is unlikely that chimerism in the central nervous system or in gametes is high enough to cause concern and, in the case of the latter, current restrictions prohibit the breeding of interspecies chimeras. Nonetheless, if the contribution to these organs reaches a problematic level, then it might be necessary to adopt 'targeted organ generation' approaches, for example by using genetic methods to restrict developmental potency (Kobayashi et al., 2015) or by transplantation of apoptosis-resistant progenitor cells with a more restricted and defined cell fate (Masaki et al., 2016).

Conclusions

Interspecies chimeric assays have the potential to advance human stem cell research by providing a highly stringent test of developmental potency and cell fate. However, there are significant barriers to their generation, and experiments must be carefully planned so as to avoid failure due to evolutionary distance and developmental mismatching. Even if these barriers can be overcome, as they likely will be in the future, the ethical and moral issues surrounding the generation of human-animal chimeras require serious consideration and investigation. At this stage, therefore, research on human-animal chimeras should be performed under absolute transparency. The current restrictions imposed on human-animal chimera research by the US National Institutes of Health and in other countries makes this difficult, however, because research instead performed using private funding is less visible to the general public than it otherwise could – and should – be.

Competing interests

The authors declare no competing or financial interests.

References

Brons, I. G. M., Smithers, L. E., Trotter, M. W. B., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S. M., Howlett, S. K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R. A. et al. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448**, 191-195.

Cohen, M. A., Wert, K. J., Goldmann, J., Markoulaki, S., Buganim, Y., Fu, D. and Jaenisch, R. (2016). Human neural crest cells contribute to coat pigmentation in interspecies chimeras after in utero injection into mouse embryos. *Proc. Natl. Acad. Sci. USA* **113**, 1570-1575.

Fehilly, C. B., Willadsen, S. M. and Tucker, E. M. (1984). Interspecific chimaerism between sheep and goat. *Nature* **307**, 634-636.

Fujiki, Y., Fukawa, K., Kameyama, K., Kudo, O., Onodera, M., Nakamura, Y., Yagami, K., Shiina, Y., Hamada, H., Shibuya, A. et al. (2003). Successful multilineage engraftment of human cord blood cells in pigs after in utero transplantation. *Transplantation* **75**, 916-922.

Gafni, O., Weinberger, L., Mansour, A. A. F., Manor, Y. S., Chomsky, E., Ben-Yosef, D., Kalma, Y., Viukov, S., Maza, I., Zviran, A. et al. (2013). Derivation of novel human ground state naive pluripotent stem cells. *Nature* **504**, 282-286.

Goldstein, R. S., Drukker, M., Reubinoff, B. E. and Benvenisty, N. (2002). Integration and differentiation of human embryonic stem cells transplanted to the chick embryo. *Dev. Dyn.* **225**, 80-86.

Han, X., Chen, M., Wang, F., Windrem, M., Wang, S., Shanz, S., Xu, Q., Oberheim, N. A., Bekar, L., Betstadt, S. et al. (2013). Forebrain engraftment by human glial progenitor cells enhances synaptic plasticity and learning in adult mice. *Cell Stem Cell* **12**, 342-353.

Hanna, J., Cheng, A. W., Saha, K., Kim, J., Lengner, C. J., Soldner, F., Cassady, J. P., Muffat, J., Carey, B. W. and Jaenisch, R. (2010). Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. *Proc. Natl. Acad. Sci. USA* **107**, 9222-9227.

Huang, Y., Osorno, R., Tsakiridis, A. and Wilson, V. (2012). In vivo differentiation potential of epiblast stem cells revealed by chimeric embryo formation. *Cell Rep.* **2**, 1571-1578.

Isotani, A., Hatayama, H., Kaseda, K., Ikawa, M. and Okabe, M. (2011). Formation of a thymus from rat ES cells in xenogeneic nude mouse↔rat ES chimeras. *Genes Cells* **16**, 397-405.

James, D., Noggle, S. A., Swigut, T. and Brivanlou, A. H. (2006). Contribution of human embryonic stem cells to mouse blastocysts. *Dev. Biol.* **295**, 90-102.

Kobayashi, T., Yamaguchi, T., Hamanaka, S., Kato-Itoh, M., Yamazaki, Y., Ibata, M., Sato, H., Lee, Y.-S., Usui, J., Knisely, A. S. et al. (2010). Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. *Cell* **142**, 787-799.

Kobayashi, T., Kato-Itoh, M. and Nakauchi, H. (2015). Targeted organ generation using Mixl1-inducible mouse pluripotent stem cells in blastocyst complementation. *Stem Cells Dev.* **24**, 182-189.

Kojima, Y., Kaufman-Francis, K., Studdert, J. B., Steiner, K. A., Power, M. D., Loebel, D. A. F., Jones, V., Hor, A., de Alencastro, G., Logan, G. J. et al. (2014). The transcriptional and functional properties of mouse epiblast stem cells resemble the anterior primitive streak. *Cell Stem Cell* **14**, 107-120.

Larochelle, A., Vormoor, J., Hanenberg, H., Wang, J. C. Y., Bhatia, M., Lapidot, T., Moritz, T., Murdoch, B., Xiao, X. L., Kato, I. et al. (1996). Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nat. Med.* **2**, 1329-1337.

Li, W., Wei, W., Zhu, S., Zhu, J., Shi, Y., Lin, T., Hao, E., Hayek, A., Deng, H. and Ding, S. (2009). Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. *Cell Stem Cell* **4**, 16-19.

Masaki, H., Kato-Itoh, M., Umino, A., Sato, H., Hamanaka, S., Kobayashi, T., Yamaguchi, T., Nishimura, K., Ohtaka, M., Nakanishi, M. et al. (2015). Interspecific in vitro assay for the chimera-forming ability of human pluripotent stem cells. *Development* **142**, 3222-3230.

Masaki, H., Kato-Itoh, M., Takahashi, Y., Umino, A., Sato, H., Ito, K., Yanagida, A., Nishimura, T., Yamaguchi, T., Hirabayashi, M. et al. (2016). Inhibition of apoptosis overcomes stage-related compatibility barriers to chimera formation in mouse embryos. *Cell Stem Cell* **19**, 587-592.

Mascetti, V. L. and Pedersen, R. A. (2016). Human-mouse chimerism validates human stem cell pluripotency. *Cell Stem Cell* **18**, 67-72.

Shultz, L. D., Schweitzer, P. A., Christianson, S. W., Gott, B., Schweitzer, I. B., Tennent, B., McKenna, S., Mobraaten, L., Rajan, T. V. and Greiner, D. L. (1995). Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J. Immunol.* **154**, 180-191.

Suzuki, N., Yamazaki, S., Yamaguchi, T., Okabe, M., Masaki, H., Takaki, S., Otsu, M. and Nakauchi, H. (2013). Generation of engraftable hematopoietic stem cells from induced pluripotent stem cells by way of teratoma formation. *Mol. Ther.* **21**, 1424-1431.

Tachibana, M., Sparman, M., Ramsey, C., Ma, H., Lee, H.-S., Penedo, M. C. T. and Mitalipov, S. (2012). Generation of chimeric rhesus monkeys. *Cell* **148**, 285-295.

Takashima, Y., Guo, G., Loos, R., Nichols, J., Ficz, G., Krueger, F., Oxley, D., Santos, F., Clarke, J., Mansfield, W. et al. (2014). Resetting transcription factor control circuitry toward ground-state pluripotency in human. *Cell* **158**, 1254-1269.

Tesar, P. J., Chenoweth, J. G., Brook, F. A., Davies, T. J., Evans, E. P., Mack, D. L., Gardner, R. L. and McKay, R. D. G. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448**, 196-199.

Theunissen, T. W., Powell, B. E., Wang, H., Mitalipova, M., Faddah, D. A., Reddy, J., Fan, Z. P., Maetzel, D., Ganz, K., Shi, L. et al. (2014). Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. *Cell Stem Cell* **15**, 471-487.

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Theunissen, T. W., Friedli, M., He, Y., Planet, E., O'Neil, R. C., Markoulaki, S., Pontis, J., Wang, H., Iouranova, A., Imbeault, M. et al. (2016). Molecular criteria for defining the naive human pluripotent state. *Cell Stem Cell* **19**, 502-515.

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.

Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J.-C., Lanzavecchia, A. and Manz, M. G. (2004). Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* **304**, 104-107.

Wu, J., Okamura, D., Li, M., Suzuki, K., Luo, C., Ma, L., He, Y., Li, Z., Benner, C., Tamura, I. et al. (2015). An alternative pluripotent state confers interspecies chimaeric competency. *Nature* **521**, 316-321.

Wu, J., Platero-Luengo, A., Sakurai, M., Sugawara, A., Gil, M. A., Yamauchi, T., Suzuki, K., Bogliotti, Y. S., Cuello, C., Valencia, M. M. et al. (2017). Interspecies chimerism with mammalian pluripotent stem cells. *Cell* **168**, 473-486.e15.

Yamaguchi, T., Sato, H., Kato-Itoh, M., Goto, T., Hara, H., Sanbo, M., Mizuno, N., Kobayashi, T., Yanagida, A., Umino, A. et al. (2017). Interspecies organogenesis generates autologous functional islets. *Nature* **542**, 191-196.

Yang, Y., Liu, B., Xu, J., Wang, J., Wu, J., Shi, C., Xu, Y., Dong, J., Wang, C., Lai, W. et al. (2017). Derivation of pluripotent stem cells with in vivo embryonic and extraembryonic potency. *Cell* **169**, 243-257.e25.

Zanjani, E. D., Pallavicini, M. G., Ascensao, J. L., Flake, A. W., Langlois, R. G., Reitsma, M., MacKintosh, F. R., Stutes, D., Harrison, M. R. and Tavassoli, M. (1992). Engraftment and long-term expression of human fetal hemopoietic stem cells in sheep following transplantation in utero. *J. Clin. Invest.* **89**, 1178-1188.