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The Fetal Sheep: A Unique Model System for Assessing the Full Differentiative Potential of Human Stem Cells

Graca Almeida-Porada, Christopher Porada, and Esmail D. Zanjani

Department of Animal Biotechnology, University of Nevada, Reno, NV, USA.

The naturally occurring stem cell migratory patterns, the availability of expanding homing and engraftment sites, and the presence of tissue/organ-specific signals in the developing mammalian fetus provide the ideal setting for stem cells to exhibit their full biological potential. These characteristics combined with the relative immunological naivete of the early gestational age fetus that permits the engraftment and long-term persistence of allogeneic and xenogeneic donor stem cells make it possible to use the developing fetus to assess the *in vivo* potential of a variety of stem cells. We have taken advantage of these permissive characteristics of the fetus to develop a large animal model of human hematopoiesis in sheep that permits not only the long-term engraftment of human hematopoietic stem cell/progenitor cells and their differentiation into the full range of lymphohematopoietic elements, but also the relatively robust expression of their potential to contribute to the formation of non-hematopoietic tissues.

Key Words: Stem cells, plasticity, in-utero transplantation, transdifferentiation, *in vivo* model

Several recent studies in animal models have convincingly demonstrated that significant numbers of functional donor-derived hepatocytes are formed within the liver of transplant recipients and can mediate the correction of a liver defect.[1-10] Other studies performed in small animal models have provided evidence that cells present within the hematopoietic system also have the ability to give rise to seemingly functional neural and muscular elements upon transplantation into recipients that possess specific molecular/cellular defects. Even in humans, indirect observations have shown that donor-derived hepatocytes, neural cells, and cardiomyocytes can arise after HSC transplantation.[11-14] This combined evidence that cells within the hematopoietic compartment have the ability to give rise to non-hematopoietic cellular elements in various tissues has raised the exciting possibility that hematopoietic stem/progenitor cells (HSC) could ultimately be used to functionally repair damaged tissues and organs in clinical settings. However, despite the exciting nature of these demonstrations of apparent cellular plasticity, an in depth understanding of the processes controlling this plasticity will be needed to delineate ways of efficiently steering the cells along a specific desired lineage, and thus harness the full therapeutic potential of these various stem cell populations. Essential for gaining this understanding is an experimental model that would allow donor stem cells to participate in the generation of cells from other unrelated tissues under normal physiological conditions, in the absence of genetic or injury-induced dysfunction within a specific organ. Ideally, such a model would also permit relatively robust formation of a wide variety of donor-derived tissue-specific cells. Because studies have now provided evidence that in the absence of selective pressure, the differentiation of bone marrow cells into other cells types such as mature hepatocytes is highly inefficient,[6,7] it is very unlikely that a healthy adult animal can fulfill these requirements.

One could argue that the complex issue of stem cell plasticity could most unequivocally be examined *in vitro*, since this would allow the investigator to carefully select which specific differentiated cell types were desired, and then closely follow the cells in culture, delineating the path-

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Reprint address: requests to Esmail D. Zanjani, Ph.D., Department of Animal Biotechnology, University of Nevada Reno, Mail Stop 202, Reno NV 89557-0104, USA. Tel: 1-775-784-7737, Fax: 1-775-784-7736, E-mail: zanjani@scs.unr.edu

ways by which the stem cells being tested adopt each alternate cellular fate. An additional consideration that would also appear to favor an *in vitro* approach is the conclusion of the scientific community at large that definitive evidence of plasticity will need to be answered by demonstrating multipotentiality of a single cell or a clonally derived cell population. For this reason, performing *in vitro* studies with highly purified populations of stem cells from various organs under highly defined culture conditions would appear to offer distinct advantages over transplantation experiments performed *in vivo* in which the experimenter has little or no control over the conditions *in vivo* within the recipient. With this said, it is not surprising that *in vitro* cultures have been pivotal in establishing the concept that adult stem cells from various tissues possess plasticity. This is especially true in the case of marrow-derived mesenchymal stem cells (MSC), likely due to the ease with which these adherent cells can be cultured and the fact that the conditions for growing many of the mesodermal cells to which MSC give rise have already been delineated. Unfortunately, this is not the case with other sources of stem cells that do not readily propagate in culture. *In vitro* studies are further complicated by the fact that the mediators required for stem cells to undergo the dramatic changes in cellular fate that have thus far been observed *in vivo* are largely unknown, making it hard to reproduce *in vitro* the conditions present within an organ microenvironment *in vivo*. It is also important to note that *in vitro* approaches are unable to duplicate conditions that affect the migratory patterns and homing of stem cells to different tissues/organs *in vivo*. Thus, at the present time, the best way in which to assess the differentiative potential of stem cells is by performing *in vivo* transplantation studies. However, since ethical and practical considerations prevent limiting dilution studies of highly defined populations of stem cells in humans, investigators have either employed animal stem cells or have been forced to test the ability of human stem cells to engraft/differentiate within a xenogeneic setting.

To date, most of the *in vivo* model systems that have been employed to demonstrate the versatility of stem cells have made use of an external stress, such as radiation-or chemical-induced injury or an experimentally created shortage of a specific cell type in the recipient to induce the transplanted cells to differentiate into the specific missing or injured organ cells.[2,9,15,16] In other studies, mdx mice that possessed a specific gene defect were used as recipients[17,18] The results of these studies have provided the valuable information that the presence of surrounding activated cells and/or signaling from the organ-specific microenvironment are essential for the transplanted cells to be induced to divide and differentiate into cells of the injured/deficient organ. However, these studies have, by nature of their design, also restricted the fate of the transplanted cells to one particular organ/system, thus preventing evaluation of the full potential of the transplanted cell populations. More recent studies in which murine NSC were microinjected into chick and mouse embryos and shown to give rise to differentiated cells of all 3 germ layers provide confirmation that previous studies in which investigators had driven the transplanted stem cells to differentiate along a single lineage had greatly underestimated the full potential of these cells.[19] However, the majority of studies that have provided evidence for stem cell plasticity have employed murine models. Since findings in mice cannot always be extrapolated to reflect the situation in higher animals and humans, a clinically relevant model is needed in which to evaluate the full potential of human stem cell plasticity.

In addition, there is another characteristic of the developing fetus that cannot be ignored. Namely, the fetus is suspended in a fluid-filled sac during development, rendering it essentially weightless, at least at the early stages of development. Given the large amount of evidence demonstrating that conditions of weightlessness (microgravity), can dramatically alter both the repertoire of genes that are expressed by numerous cell types and the ability of *in vitro* grown cells to accurately reiterate their *in vivo* functionality, it is reasonable to suppose that the weightless state of the early gestational fetus may not only play a role in the normal process of embryonic/fetal development, but may also make the fetal recipient a unique model system in which transplanted stem cell populations can express their full range of

plasticity, potentially adopting alternate cellular fates that have as yet not been observed *in vitro* or in other *in vivo* adult model systems.

The Rationale for Using a Fetal Model to Assess HSC Plasticity

The understanding of the processes that regulate the events controlling the formation of functional tissues during ontogeny is still in its infancy. Conventional teaching held that an entire multicellular organism was produced from a single totipotent stem cell that underwent multiple cycles of division/differentiation during the complex process of embryonic development, with these original stem cells shedding some of their totipotency with each subsequent division, thereby committing to a certain tissue and/or cellular lineage. It was thought that each one of the three unique germ layers formed during embryogenesis was responsible for the formation of specific mature tissues such that brain and skin were of ectodermal derivation, blood and muscle were mesodermal, and intestine and hepatocytes were formed from endoderm. This led to the reasonable assumption that further development of these tissues and their repair when necessary stemmed from precursor elements with established commitment to the tissue type involved. In support of this supposition are studies showing that adult tissues like skin,[20] mammary gland[21] and muscle[22] all contain regenerative stem cells. The adult mammalian brain once thought incapable of regeneration was found not only to contain self-renewing multipotent stem cells but also to produce new neurons throughout adult life,[19,23,24] and mature hepatocytes were found to be able to revert from their mature form to a more primitive phenotype and adopt a broader differentiative potential in the presence of appropriate growth factors and matrix signaling.[25] In each of these cases, it was presumed that these stem cells were still essentially tissue-specific and could only contribute to the formation of cells from embryologically related organs. However, a number of recent studies have provided evidence that stem cells isolated from several tissues have the ability to give rise to cells of other seemingly unrelated organs, suggesting that this presumption may not be valid,[11,16,18] and thus challenging the conventional dogma that cellular differentiation and lineage commitment are irreversible processes. This apparent plasticity of these adult stem cells opens new fields of stem cell research, since although at this point the limited characterization of these cells prevents appreciation of their full utility, scientific proof of their pluripotency would clearly establish them as likely candidates for multiple therapeutics. While the mechanisms underlying such a change in cell fate are at present unknown, as are the precise cell types responsible, it is clear that for proper function the cells must reach the target organ. The circulatory system provides an efficient stem cell distribution system throughout life. During fetal life, a series of well-established migratory processes likely employing the circulatory system insure that adequate numbers of appropriate stem/progenitor cells reach the target tissues/organs when needed. Under the permissive milieu of the target organ, these cells function to produce the required type(s) of cells. The existence of this highly permissive milieu is very likely associated with the continuous need for new cells during fetal development. For these reasons, we hypothesized that the ideal way to evaluate the full plasticity of human stem cells would be to transplant human stem cells into fetal recipients at a point in development when all the organs had begun to differentiate but the need for exponential growth and differentiation could still permit the possibility of reprogramming of cellular fate through a bombardment of proliferative/differentiative stimuli without forcing the transplanted cells to adopt a single specific fate by damaging/inducing regeneration within one particular organ. Since transplantation of the human donor cells is performed during the fetal period in which all of the organs are rapidly proliferating and differentiating, the transplanted cells should thus be provided with the opportunity to find the right stimulus in each organ to give rise to different cells, assuming of course, that the transplanted cells harbor that potential. If the supposition that the appropriate microenvironmental influence can induce a cell with a mature phenotype to regress into an undifferentiated state, and/or a primitive

stem cell to start differentiating into a new lineage, then the fetus should represent an ideal model system in which to examine the full potential of an adult stem cell.

In addition, there is another characteristic of the developing fetus that cannot be ignored. Namely, the fetus is suspended in a fluid-filled sac during development, rendering it essentially weightless, at least at the early stages of development.[26-29] Given the large amount of evidence demonstrating that conditions of weightlessness (microgravity), can dramatically alter both the repertoire of genes that are expressed by numerous cell types[30-35] and the ability of *in vitro* grown cells to accurately reiterate their *in vivo* functionality,[36-37] it is reasonable to suppose that the weightless state of the early gestational fetus may not only play a role in the normal process of embryonic/fetal development, but may also make the fetal recipient a unique model system in which transplanted stem cell populations can express their full range of plasticity, potentially adopting alternate cellular fates that have as yet not been observed *in vitro* or in other *in vivo* adult model systems.

The Fetal Sheep Model of Human Stem Cell Transplantation

We have taken advantage of these permissive aspects of the developing early gestational age pre-immune fetus to develop a large animal model of human HSC plasticity in sheep.[38-44] For many reasons, the sheep fetus represents an ideal model system in which to explore stem cell transplantation and plasticity. The large size of the sheep and its relatively long life span allows both the evaluation of donor cell activity in the same animal for years after transplant and allows the investigator to easily obtain sufficient human cells from the primary recipients to perform serial transplantation; experimental opportunities that are not easily possible with murine models. Indeed, successful engraftment and multilineage differentiation of human hematopoietic stem cells has now been observed in primary, secondary, and tertiary recipients using this model system. In addition, the sheep fetus shares many important physiological and developmental characteristics with the human fetus, and the large size of the sheep fetus enables manipulation early in gestation. Procedures for gaining and maintaining access to the sheep fetus are well established. Fetal sheep have been used extensively in the study of mammalian fetal physiology, and results obtained with this model have been directly applicable to the understanding of human fetal growth and development,[45] and have been highly predictive of clinical outcome following in utero HSC transplantation. Furthermore, the development of the ovine immune system during gestation has been extremely well characterized,[46-49] making the sheep an ideal model in which to examine the immunologic aspects of in utero transplantation. Importantly, in contrast to other model systems for the study of stem cell transplantation, the fetal sheep recipient has a normal functioning immune system, but is still able to support the engraftment/differentiation of human HSC if the transplant is performed at the appropriate stage of fetal life. In early immunologic development, before thymic processing of mature lymphocytes, the fetus appears to be largely tolerant of foreign antigens.[50,51] Furthermore, exposure to foreign antigens during this period often results in sustained tolerance, which can become permanent if the presence of antigen is maintained.[52,53] By taking advantage of this so-called "window of opportunity" and performing the transplant during the "pre-immune" stage of development, significant levels of engraftment are possible in the absence of irradiation or other myeloablative therapies. In addition to these immunologic advantages, the existence of the naturally occurring migratory patterns in the fetus facilitates the widespread efficient distribution of donor HSC throughout the body. Once there, the cells are influenced by the stimulatory environment of the specific tissue/organ to undergo proliferation and directed differentiation. In this model, human HSC derived from fetal liver, fetal bone marrow, cord blood, adult bone marrow, and mobilized adult peripheral blood all readily engraft and undergo multilineage differentiation. Because of the absence of any myeloablative conditioning, transplanted human HSC must compete with the endogenous HSC for available niches within the

bone marrow, suggesting that the events that occur following transplantation of human HSC into the sheep fetuses may be biologically relevant to events in humans.

Plasticity of Human Cells in the Fetal Sheep Model

The versatility of this non-injury fetal model for the study of human stem cell plasticity was demonstrated by its ability to reveal the differentiative potential of different populations of both human HSC and human mesenchymal stem cells (MSC). When highly purified populations of human HSC from bone marrow and mobilized peripheral blood were transplanted into the fetal sheep model, they gave rise to human mesenchymal stem cells providing evidence that this model was at least capable of supporting the switch of the transplanted cells from one mesodermal fate to another.[54] We have also shown in other studies that enriched populations of human adult bone marrow and umbilical cord HSC have the ability to generate functional human hepatocytes within the developing fetal liver.[55] Importantly, this trans-differentiation from mesodermal HSC to endodermal hepatocytes occurred in the absence of any injury or stimulus, underscoring the importance of the micro-environmental influence of a fetal milieu in the realization of the full potential/plasticity of adult stem cell populations.

Recently we examined the ability of human fetal brain-derived stem cells (NSC) to contribute to blood cell production after transplantation in our model. Human NSC were able to produce not only differentiated cells of multiple hematopoietic lineages but also CD34+ cells that were able to repopulate secondary transplant recipients. While further analyses revealed apparent qualitative differences between the hematopoietic cells derived from NSC as compared to those derived from marrow HSC, our findings demonstrate that human fetal brain-derived NSC harbor the ability to respond to the microenvironmental cues present within the intact fetal hematopoietic system and reprogram their differentiative agenda, generating both a range of differentiated hematopoietic cells types and HSC that are capable of engraftment upon serial transplantation.[56,57] We are currently using the fetal sheep model to analyze differences between phenotypically distinct human HSC with respect to their plasticity, and addressing possible differentiative pathways that allow for the conversion of human HSC to hepatocytes.

Bone marrow mesenchymal stem cells have been shown in several different models to have the ability to differentiate into a number of other different cells such as chondrocytes, adipocytes, myocytes, endothelium, as well as cells of alternate germinal derivation such as neural cells, skin, and liver, suggesting that MSC likely represent one of the most promising stem cell sources for tissue replacement therapy.[58-65] For these reasons, we evaluated the ability of these human cells to give rise to other cell types *in vivo* using the fetal sheep model. To this end, we isolated several clonal MSC populations from adult BM and evaluated their ability to give rise to donor (human)-derived blood and other organ-specific cell types. All of the transplanted MSC clones generated multilineage hematopoietic cells including CD34+ cells. Analysis of livers from these animals revealed some of the clones were capable of giving rise to significant numbers of human hepatocytes, detected by both immuno-histochemistry using a monoclonal antibody specific for human hepatocytes and in situ hybridization using a human Alu-specific probe. Examination of skin from these same sheep demonstrated the presence of human keratinocytes expressing human-specific cytokeratin. These findings were confirmed by in-situ hybridization using a human Alu-specific probe. Through these studies, we were able to demonstrate that the sheep model also supports the differentiation of clonal populations of BM-derived stem cells into cells of all three germinal derivations.[66] We also examined the ability of our model to support the differentiation of MSCs derived from tissues other than BM. We reasoned that the fetal kidney might represent a rich source of pluripotent mesenchymal stem cells, based on both the mesenchymal origin of the kidney and the role played by the developing mesonephros in primitive hematopoiesis.[14] To test this hypothesis, fetal kidney

MSCs were transplanted into pre-immune fetal sheep and recipients were evaluated for the presence/engraftment of human cells beginning at 2 months post-transplant and at intervals thereafter until 9 months post-transplant. At 2 months post-transplant, human hematopoietic cells were readily detectable in the hematopoietic system of the transplanted sheep and the sheep maintained their chimeric status for at least 9 months. We also looked at the ability of these cells to give rise to human hepatocytes in our model. Sections were first analyzed with an antibody to human hepatocytes. Using this approach, human hepatocyte-like cells could readily be detected within the sections from sheep transplanted in utero with the human metanephric mesenchymal cells. In order to demonstrate that the hepatocyte-like cells generated were functional, sections from the liver of these same animals were analyzed by immunohistochemistry with an antibody specific for human albumin. Human hepatocyte-like cells present within the liver sections stained positively with this albumin antibody, demonstrating that the cells generated from the metanephric mesenchymal cells are functional. Interestingly, the levels of human hepatocyte-like cells appeared to correlate well with the levels of human hematopoietic cell engraftment, with animals containing higher levels of human hematopoietic cells exhibiting greater numbers of human hepatocyte-like cells within their liver. In all of the sections staining positive for human hepatocyte-like cells, the human origin of the hepatocyte-like cells was confirmed by in situ hybridization with a human-specific Alu probe. Recently controversy has arisen regarding the origin of donor liver cells post-transplantation and several investigators have shown that cell fusion is at least one of the possible mechanisms responsible for the apparent differentiation of hematopoietic cells into liver cells.[67] Our sheep model allows the robust formation of relatively large numbers of human-derived hepatic cells under normal physiologic conditions, and could thus provide a valuable tool for the study of the mechanisms underlying the involvement of cells of human hematopoietic compartments in hepatopoiesis.

In conclusion, the results obtained thus far with the fetal sheep model suggest that this xenogeneic system is ideally suited to the study of the *in vivo* behavior and plasticity of human stem cells from various tissues following transplantation, by virtue of its ability to permit the expression of such a degree of human stem cell plasticity in the absence of injury or induced regeneration within a specific tissue. Furthermore, the versatility of the pre-immune fetal sheep may be useful in the isolation, characterization, and evaluation of tissue/organ-specific human stem/progenitor cells, and in exploring the possibility of generating patient-specific cells/tissues/organs for purposes of transplantation.

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