Establishment of Transplantable Porcine Tumor Cell Lines Derived from MHC Inbred Miniature Swine

Running Title: Transplantable Porcine Tumor Cell Lines

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Abstract

The lack of transplantable tumors has limited assessment of graft versus tumor effects following hematopoietic cell transplantation in clinically relevant large animal models. We describe the derivation and characterization of porcine tumor cell lines with initial efforts of tumor transplantation using immunocompromised mice and highly inbred sublines of MGH MHC-inbred miniature swine. Autopsies were performed routinely on swine that died unexpectedly or had suspicion of malignancy based on clinical symptoms or peripheral blood analysis. Tissue samples were obtained for pathology, phenotyped by flow cytometry, and placed in culture. Based on growth, lines were selected for passage into NOD/SCID mice and miniature swine. Porcine tumor recipients were pre-conditioned with total body irradiation from 0 to 500cGy or with a 30-day course of oral cyclosporine. We identified nineteen cases of hematological tumors. Nine distinct tumor cell lines were established from eight of these cases, including three derived from highly inbred sublines. In vivo tumor growth and serial transfer was observed in immunocompromised mice for one tumor cell line and in miniature swine for one of two tumor cell lines expanded for this purpose. These results suggest the possibility of developing a transplantable tumor model in this large animal system.
Introduction

Animal models of malignancy are valuable tools for studying cancer biology. Although rodent models have aided in elucidating mechanisms of oncogenesis and in developing treatment strategies, small animal systems are limited in their ability to accurately reflect all aspects of the disease conditions associated with human cancers.\(^1\,^2\) Translation from these small animal models towards the clinical setting is not always possible, as humans often have different responses and disease mechanisms.\(^3\,^4\) Since the life span of these animals is significantly shorter, it is also difficult to utilize them for longitudinal studies to investigate the efficacy and safety of new treatments.\(^5\,^6\) Large animals with greater genetic and physiologic similarities to humans can be used to bridge this translational gap.\(^7\) Such models of malignant diseases rely on spontaneous neoplasms occurring in certain breeds, such as canine melanoma, or on induced tumors resulting from exposure to a carcinogen.\(^7\,^9\) Virally induced tumor cells have also been used for evaluating imaging modalities and feasibility of interventional radiological techniques in dogs, but these systems may not necessarily resemble \textit{in situ} malignancies of those organs.\(^10\) The low incidence of disease and the length of time to disease onset limit practicality of these models for experimentation.

Non-myeloablative HCT protocols that achieve potent graft versus tumor (GVT) effects without graft versus host disease (GVHD) have been developed in rodent models.\(^11\,\!^13\) Successful translation of these protocols to the clinic has been limited, however, with GVHD remaining a major complication. Our laboratory has developed minimally toxic HCT protocols in miniature swine that achieve stable engraftment across MHC barriers without GVHD.\(^14\) The lack of available transplantable tumor lines in miniature swine has so far prohibited the direct evaluation
of anti-tumor effects of HCT and donor leukocyte infusion (DLI) in this clinically relevant large animal model.

Recently, histocompatible sublines of SLA\textsuperscript{dd} animals have been established that accept reciprocal skin grafts.\textsuperscript{15} Isolation of tumors and establishment of tumor cell lines from these animals should permit development of transplantable tumor models to assess GVT effects in miniature swine. Over the past ten years, we have monitored our herd for the occurrence of malignancies, particularly hematologic cancers such as leukemias and lymphomas. From this surveillance, we now report our establishment of porcine tumor lines and our initial efforts of tumor transplantation.

**Methods**

**Animals**

MGH partially inbred MHC-defined miniature swine have previously been described in detail.\textsuperscript{16,17} Three lines are homozygous for a different MHC haplotype (SLA\textsuperscript{a}, SLA\textsuperscript{c} and SLA\textsuperscript{d}), and five lines bear different intra-MHC recombinant haplotypes (SLA\textsuperscript{f}, SLA\textsuperscript{g}, SLA\textsuperscript{h}, and SLA\textsuperscript{k}). Sublines of the SLA\textsuperscript{dd} animals were established by sequential brother-sister matings to produce histocompatible lines with a coefficient of inbreeding of >94\%. Reciprocal skin grafts within these sublines were accepted without immunosuppression.\textsuperscript{15} All animal care procedures were in compliance with the *Principles of Laboratory Animal Care* and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (publication No. 86-23, revised 1985). Protocols involving
animals were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Detection and Diagnosis

Animals were under continuous surveillance for the development of abnormal symptoms and physical findings. The preliminary screen for malignancy included review of clinical symptoms, physical findings, complete blood count (CBC), and white blood cell (WBC) differential by manual count and flow cytometry. Based on this screen, animals suspected of malignancy were sacrificed and underwent an autopsy under aseptic conditions to obtain primary disease tissues. Animals that died unexpectedly were also subject to autopsy under aseptic conditions for tissue harvest and for diagnostic purposes. The diagnosis of malignancies was based on the clinical findings, history, physical examination, CBC, histology, and phenotypic analysis by surface staining and flow cytometry of peripheral blood cells.

Tissue and Peripheral Blood Mononuclear Cell Harvest and Processing

At the time of autopsy, tissue samples from lymphoid, hematopoietic, and any organ that appeared abnormal at autopsy were obtained in a sterile manner. Samples were then processed by mechanical maceration and subsequent filtering with Hanks Buffered Salt Solution (HBSS) through a 40um cell strainer (Becton Dickinson). Peripheral blood was processed for mononuclear cells (PBMC) from heparinized whole blood diluted with HBSS containing Ca$^{2+}$ and Mg$^{2+}$. Cells were obtained by gradient centrifugation using lymphocyte separation medium (ICN Biomedicals, Inc., Aurora, OH) and lysis of red blood cells with ACK Lysing buffer (Cambrex BioScience, Walkersville, MD)
Histology and Immunohistochemistry

At necropsy, any grossly abnormal tissue and suspicious lesions were sampled for histology and immunohistochemistry. For histology, tissues were fixed in paraformaldehyde, and then stained using hematoxylin and eosin. For immunohistochemistry, tissues were embedded in Histoprep embedding matrix (Fisher) and frozen at -80°C. The following primary antibodies were used for immunohistochemistry: CD3 BB23-8E6 IgG1\(^{18,19}\); CD16 G7 IgG1\(^{20}\); and CD172a (porcine monocyte/granulocyte marker) 74-22-15 IgG1\(^{21}\).

Tissue Culture

Cell suspensions obtained from processing were placed into culture with RPMI 1640 media supplemented with 12% fetal bovine serum (FBS), 10 mM HEPES, 1 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 2x10\(^{-5}\) M 2-mercaptoethanol. Cultures are maintained at 37°C with 5% CO\(_2\). Establishment in culture was determined by continuous growth over a minimum period of two months. Once cell lines were established, each line was maintained with passage every 7 to 14 days. To identify growth parameters of the established cell lines under these conditions, cells were plated at an initial density of 1x10\(^4\) cells/ml in a T75 flask (Corning). Daily cell counts were obtained over a 2 week incubation period. Samples of cell lines were collected and frozen at -180°C in liquid nitrogen at a concentration of 1x10\(^7\) cells/ml in cryoprotective medium (Cambrex BioScience, Walkersville, MD) diluted 1:1 with FBS to a final concentration of 7.5% DMSO.

Flow Cytometry
Peripheral blood samples obtained at the time of clinical symptoms were analyzed for a preliminary phenotype by flow cytometry. Cell suspensions from primary tissues including processed PBMC, lymph node, liver, bone marrow and spleen, as well as established tumor cell lines were also phenotyped using flow cytometry. Antibodies used included the following: CD1 76-7-4 IgG2a\(^{21}\); CD2 MSA-4 IgG2a\(^{22,23}\); CD3 898H2-6-15 IgG2a\(^{24}\); CD4 74-12-4 IgG2b\(^{21}\); CD5 BB6-9G12 IgG1\(^{25}\); CD8 76-2-11 IgG2a\(^{26}\); CD9 1038H-4-6 IgM\(^{27}\); CD16 G7 IgG1\(^{20}\); CD21 BB6-11C9 IgG1\(^{28,29}\); CD25 231.3B2 IgG1\(^{30}\); CD172a 74-22-15 IgG1\(^{21}\); class II DR 40D IgG2b; anti-mu heavy chain 5C9 IgG1\(^{31}\); and anti-k light-chain K139 3E1 IgG2a\(^{32}\). Flow cytometry was performed using a Becton Dickinson FACScan (San Jose, CA). Staining of cell suspensions was performed as previously described\(^{33}\). Data were analyzed using Winlist list mode analysis software (Verity Software House, Topsham, ME).

In vivo transfer of tumors into NOD/SCID mice

In an attempt to select for variants with in vivo growth potential, cultured tumor cell lines were transplanted into NOD/SCID mice (Jackson Laboratories, Bar Harbor, ME). Animals were 4-8 weeks of age and were housed in clean conditions. Animals were manually restrained and steriley swabbed with a 70% alcohol solution at the site for intraperitoneal (IP) injection in the lower right abdominal quadrant. Sterile 23 to 27 gauge needles were used to deliver the sterile inoculum of tumor cells at a dose of ranging from 1-5 x 10\(^7\) cells in a total volume of 0.2ml. Tumor tissue was removed steriley to obtain samples for cell culture, pathology, and surface phenotyping by flow cytometry. These tissue samples were also processed by mechanical maceration and subsequent filtering with Hanks Buffered Salt Solution through a 40um cell
strainer (Becton Dickinson). Cell suspensions were placed in culture, under the conditions above.

*In vivo transfer of tumors in miniature swine*

Tumor cell lines established from highly inbred SLA$^d$ animals were used for *in vivo* tumor transfer experiments using recipient animals also within this subgroup. Cells were injected into animals within histocompatible sublines, when possible, and also across sublines that share a common ancestor at the fifth generation of inbreeding. The degree of coancestry was calculated between the recipient and the animal from which the tumor cell line was derived. Recipient animals received tumor cell injections at doses from $1 \times 10^7$ to $1 \times 10^8$ cells/kg intravenously (IV) and/or subcutaneously (SC) multiple injection sites and from $1 \times 10^7$ to $1 \times 10^8$ cells in up to four individual injection sites on the rear flanks of the animal, at up to four sites. Recipient animals ranged in age between 1.5 to 4 months of age and in weight between 5kg to 20kg in size. These animals were prepared with total body irradiation (TBI), a 30 day course of cyclosporine A (CyA), or no additional treatment. Animals receiving TBI prior to cellular injection were irradiated day -2 with 100, 300, or 500 cGy of TBI. Animals treated with concurrent CyA received Neoral (Novartis Pharmaceuticals, East Hanover, NJ) beginning on day -1 and continuing for 30 days. Dosages were started at 15mg/kg on day -1, and adjusted to maintain CyA whole blood levels between 400-800 ng/mL during the treatment period. Animals were monitored for tumor growth by palpation of injection sites. Blood samples from animals were analyzed for a complete blood count and for tumor growth after IV tumor administration by flow cytometry. If tumor growth was detected, animals were euthanized. Tissues were steriley harvested and processed for analysis and for culture, as outlined above.
Results

Incidence of Malignancies in MGH MHC-Defined Miniature Swine

From surveillance of our herd, we identified nineteen cases of malignancies. Ten of these cases were derived from animals with post-transplant lymphoproliferative disease (PTLD), a complication of either experimental hematopoietic cell or solid organ transplant protocols. The incidence of PTLD occurring in these protocols has been previously reported. Since no experimental HCT recipients from the inbred sublines were used, all PTLD cases occurred outside of the highly inbred SLA\textsuperscript{dd} sublines of animals. The remaining nine cases were spontaneously occurring malignancy. Of these cases, five occurred within highly inbred SLA\textsuperscript{dd} histocompatible sublines. To determine the incidence of spontaneous, not transplant-related malignancy, we utilized our animal database consisting of data collected over the last ten years and evaluated the number of cases occurring in adult naïve animals greater than or equal to 24 months of age and unassigned to an experimental protocol. As most animals in our herd do not reach 24 months due to their use in experiments, only these animals were considered relevant for comparison. The overall incidence of spontaneous malignancy among susceptible animals (≥24 months of age) within these sublines was 2.5% (4/159), while disease incidence among all other haplotype lines was 1.3% (4/301), giving a p=0.64 (χ\textsuperscript{2}=0.216). A similar comparison of disease in incidence in older populations (≥36 months) revealed a statistically significant incidence of 10% (4/40) in the histocompatible sublines versus 1.3% (3/223), giving a p=0.016 (χ\textsuperscript{2}=5.763). However, it is uncertain whether this is an accurate reflection of disease incidence in our miniature swine as there are a limited number of animals, particularly for the SLA\textsuperscript{dd} subline, that reach this age.
Diagnosed Malignancies Observed in Miniature Swine

The age, haplotype, and diagnosed malignancy of animals that developed disease are compiled in Table 1. After the fifth generation (G5) of brother-sister mating, three parallel sublines with common ancestry at G5 were set up for further inbreeding. For the purposes of this study, we will refer to these distinct sublines as DD1, DD2, and DD3. For disease occurring in highly inbred SLA<sup>dd</sup> animals, the subline to which each animal belonged is also noted in Table 1. Of the nineteen observed cases of malignancy, six cases were diagnosed as leukemia, eleven were lymphomas, and one was a non-identified carcinoma with lung involvement. All cases of leukemia occurred in adult animals ranging from 30 to 76 months at time of disease diagnosis. Three cases occurred in inbred SLA<sup>dd</sup> animals and characterization of disease has been concurrently reported (Cho et al., submitted for publication). Only one case of lymphoma was a spontaneous neoplasm, which occurred in an inbred SLA<sup>dd</sup> adult animal 38 months old. The remaining ten cases of lymphoma were due to post-transplant lymphoproliferative disease (PTLD).<sup>34-36</sup> These cases occurred in adolescent animals between 3 to 7 months of age.

Clinical Presentation and Laboratory Findings of Animals at Initial Presentation

Animals that developed malignancies, whether spontaneously occurring or secondary to transplantation, demonstrated similar clinical presentations. On initial presentation, animals exhibited lethargy, pallor, decreased appetite, and weight loss. Laboratory analysis of animals was significant for elevated white blood cell counts (WBC). Animals with lymphoma or PTLD had elevated WBC greater than 30,000 cells/μL, while those eventually diagnosed with leukemia typically had counts greater than 50,000 cells/μL. The peripheral blood of most animals with
spontaneous lymphoma or PTLD showed elevated B-cell counts greater than 2,000 cells/μl, with the presence of enlarged blast-like cells in the peripheral blood. By similar analysis of animals with leukemia, flow cytometry analysis was significant for the presence of enlarged blast-like cells in the peripheral blood. These cells were determined to be myeloid in origin based on positive surface staining of markers CD16 and CD172a. The animal that developed a carcinoma demonstrated similar clinical symptoms including fever, decreased appetite, and weight loss. This malignancy was histologically diagnosed as a non-specific carcinoma obtained from the lung at necropsy.

**Gross Anatomic and Histologic Findings of Primary Tumors**

In animals with lymphoma or PTLD, mild hepatosplenomegaly and diffuse lymphadenopathy which included prominent enlargement of mesenteric lymph node chain was observed at autopsy (Figure 1a). This lymphadenopathy, in some cases, also included significantly enlarged lymph nodes in the neck and inguinal regions of the animal. Lymphoid tissues had histological evidence of atypical cells and destruction of normal architecture (Figure 1b). Animals with leukemia had less significant lymphadenopathy and greater hepatosplenomegaly, with firmer and paler organs (Figure 2a). Lesions 0.5 to 1cm in diameter were commonly found on the liver of animals with leukemia. Pleural effusions and ascites were not uncommon in these animals. There was also histological evidence of diffuse infiltration consisting of large, pleiomorphic malignant cells containing heterochromatric nuclei. These cells were present throughout all vascularized tissues such as the kidney and the lung, and they were also prominent in the bone marrow (Figure 2b).
Establishment of Tumor Cell Lines

Of the nineteen cases of malignancy, tissues from eight animals grew into tumor cell lines. Since two phenotypically distinct cell lines were derived from lymph node (LCL-17016L) and PBMC (LCL-17016P) from animal 17016, these eight cases allowed for the establishment of nine distinct tumor cell lines. Characteristics of each line, including cell surface markers and doubling time, are summarized in Table 2, while *in vitro* growth patterns are shown in Figure 3. All established tumor cell lines express surface MHC class I (dull) and MHC class II in addition to the pertinent markers listed in the last column of Table 2. Each of the five lymphoma lines was established after obtaining primary tumors from involved lymph nodes and/or processed PBMC and placing these cells in culture. Tumor lines were considered established once they were able to be passaged continuously in culture for a minimum period of two months prior to freezing down for storage. The leukemia lines were established from cultures of either processed bone marrow or processed PBMC. Leukemia cell lines had positive staining for both myeloid markers CD16 and CD172a, which similar to pattern of cell surface markers on the primary tumors originally identified in diseased animals. Cell lines derived from lymphoma or PTLD lacked CD3 and CD5 found on T-cells and myeloid markers such as CD16 and CD172a. One line, LCL-17018, gained expression of myeloid marker CD172a after *in vitro* culture. With this one exception, the pattern of cell surface markers was similar to the primary tissues from which the lines were derived.

In Vivo Tumor Growth After Transfer into Immunocompromised Mice

To select variants with better *in vivo* growth potential, three tumor cell lines were initially tested for *in vivo* growth after transfer into NOD/SCID mice. The three tumor cell lines tested included
LCL-13271, ML-12933, and CML-14736. Results of these experiments are summarized in Table 3. Of these lines, only LCL-13271 demonstrated growth in vivo after intraperitoneal injection into mice (Figure 4). This particular cell line was isolated from a SLA_{ad} animal 13271, that developed recipient-derived PTLD after a hematopoietic cell transplant.\(^{34}\) Similar to the phenotype of some human lymphoma cell lines,\(^{37}\) LCL-13271 had a stable phenotype after 72 passages based on continued expression of CD2 and CD25 while lacking other T cell markers such as CD3 and CD5 and lacking myeloid markers such as CD16 and CD172.

LCL-13271 cells were initially injected intraperitoneally into four NOD/SCID mice at a dose of 1.5x10\(^7\) cells. After two months, animals had palpable masses that were harvested and evaluated by culture and histology. Abnormal cells with pleiomorphism and heterochromatic nuclei were found and were similar to what was seen in the primary tumor (Figure 4). Cell suspensions from these tissues were confirmed as LCL-13271 in origin based on expression of CD2 and on the lack of other T cell and myeloid markers. For secondary transfer, these cells were then injected into three additional NOD/SCID mice at a dose of 5x10\(^7\) cells, and these animals also grew palpable tumors. Tissues were harvested, processed, cultured, and frozen.

**In Vivo Tumor Growth After Serial Transfer in Miniature Swine**

Our initial efforts of in vivo experiments in miniature swine focused on tumors and recipient animals from within the highly inbred histocompatible subline of SLA_{dd} miniature swine. Although LCL-13271 demonstrated growth in immunocompromised mice, we did not test this particular line in miniature swine initially since it was derived from a SLA_{ad} animal. Two established cell lines from SLA_{dd} inbred animals – CML-14736 and ML-12933 – were expanded
in culture and transplanted into miniature swine (Table 3). Of these two lines, only CML-14736 demonstrated \textit{in vivo} growth after intravenous and subcutaneous injection into irradiated miniature swine (Figure 5). This particular line was established from the bone marrow cells from animal #14736 that was diagnosed with chronic myeloid leukemia. CML-14736 has been maintained in culture for over twenty passages and maintains the phenotype of the original tumor with surface expression of myeloid markers CD16 and CD172a and with a lack of T-cell markers CD3 and CD5.

CML-14736 did not grow in animals that received only CyA or were pre-treated with 100cGy or less TBI. Pre-treatment with 500cGy TBI allowed for growth after both intravenous and subcutaneous tumor administration, while 300cGy TBI was sufficient for growth following subcutaneous growth (Table 4). All tumor growth occurred within two weeks post-injection. Animals that grew tumors after receiving SC injections developed palpable masses at the injection site (Figure 5a). In one case of tumor growth following IV administration, abnormal malignant cells could be detected in the lung (Figure 5b). Tissue sampled from these masses revealed abnormal cells with pleiomorphism and heterochromatic nuclei, suggestive of a neoplastic process, and also appeared similar in morphology to cells found in the primary bone marrow tissue of animal #14736. These cells seemed to be actively dividing based on the presence of mitotic figures. There was a lack of a cellular infiltrate, suggesting that the recipient did not mount an immune response to these cells. Immunohistochemistry of these tissues revealed positive staining for myeloid markers CD16 and CD172a, with negative staining for T cell marker CD3 (Figure 5c). These findings were consistent with the phenotype of cultured CML-14736 cells. Cell suspensions were made from these lesions and had a phenotype identical
to the CML-14736 cultured cells. Secondary transfer by SC injection of these cells into an animal pre-treated with 300cGy TBI led to growth of a palpable mass injection site.

Discussion

We have now identified and diagnosed nineteen cases of malignancies in miniature swine, including five cases within animals used to develop histocompatible sublines. With the exception of one case all were hematologic malignancies, either leukemias or lymphomas. The cases of lymphoma and leukemia were similar to human disease (Cho et al, manuscript in preparation). The incidence of post-transplant malignancy is high and restricted to certain haplotypes due to experimental protocols, and these results have been previously reported. Overall incidence of spontaneous malignancy in our herd is low, but appears to be slightly increased in the highly inbred SLA<sup>dd</sup> sublines. However, this difference is not statistically significant when considering all animals ≥ 24 months of age and may reflect our increased awareness and lower threshold for suspicion of malignancy. There may also be a predilection for disease within this group, therefore suggesting a possible genetic etiology.

These malignancies have allowed us to successfully establish nine tumor cell lines, including three lines derived from highly inbred SLA<sup>dd</sup> animals. Our experience with tumor cell lines from these cases of malignancy is similar compared to the published experience of human tumor lines. Human cell lines are typically considered established after six months of continuous growth and demonstrate immunophenotypic markers similar to their primary tumors. For these porcine tumors, a minimum period of two months appeared to be sufficient to allow for in vitro tumor cell growth and establishment of a line. Although this is shorter than the time to establish human
tumor lines, frozen aliquots of our porcine tumors from this stage in vitro could be thawed and be easily grown continuously in culture.

Similar to human tumors, porcine tumor cell lines could not be derived from all cases of malignancy. In published reports, success rates for establishment of cell lines derived from human primary leukemia and lymphomas range between 10-20%. Growing new tumor lines from hematologic malignancies is largely an unpredictable random process, though several reasons have been suggested to explain the failure of in vitro culturing. One explanation is the removal of these neoplastic cells from original environment. It has been suggested that this change may alter factors contributing to their growth, such as support from hematopoietic growth factors. In vitro cultures may lack these elements and lead to the death of neoplastic cells, while promoting overgrowth of non-neoplastic cells. This overgrowth of other types of cells was observed in our lymphoma cultures and may have prevented establishment of a cultured line. The removal from their native environments may also alter beneficial interactions, such as with supporting cells such as fibroblasts. In porcine leukemia lines, growth of a fibroblast-like layer was observed in cultured primary tissue and may have contributed to the establishment of some lines.

The tissue source of primary tumor cells may influence the culture of leukemia and lymphoma lines. Most human lines have reportedly been derived from ascites, processed peripheral blood or bone marrow samples, rather than from solid tissues such as lymph nodes. This may partly be due to the limited access of these tissues in the clinic. The differences in culturing tissues may also be due to the clinical status of the patient at the time tumor tissue is obtained. For example,
leukemia cell lines were more likely to be established when tissues were obtained from CML patients during a blast crisis when the tumor burden is greater and there is decreased immune surveillance.\textsuperscript{39} Our relative success in culturing porcine leukemia lines may be attributed to harvesting tissues during a blast crisis when clinical symptoms were prominent and prompted sacrifice of the animal. In these instances, cell suspensions of processed blood and bone marrow may have been more homogeneous for abnormal cells. For tissues which did not grow into tumor lines, primary cell suspensions may have been heterogeneous containing both abnormal neoplastic cells and reactive T-cells. This heterogeneity would reflect a lower tumor burden and possibly greater immune competence of the host animal.

Established porcine tumor cell lines have multiple applications, including a range of \textit{in vitro} uses in investigating oncogenesis and in the development of biological assays and tools.\textsuperscript{2,41,42} One particular application of interest of these lines is the development of pre-clinical large animal transplantable tumor models using histocompatible miniature swine. Tumor transplantation in syngeneic rodent systems has been widely utilized for modeling cancers.\textsuperscript{43,44} Initial attempts of tumor transplantation using our cell lines in histocompatible miniature swine led to successful growth in cases where recipients were pre-treated with irradiation. However, we have not yet achieved tumor growth without immunosuppression. One possible explanation is the pattern of surface marker expression. Markers such as MHC antigens can lead to rejection by the host immune system. Although this risk is minimized by matching of major and many minor histocompatibility loci, there may be other minor or unknown antigens present which can still be recognized as foreign by the host immune system.\textsuperscript{45-48} Conversely, a lack of antigens or surface markers may also contribute, as markers necessary for \textit{in vivo} growth of our porcine tumor lines
may be lost by \textit{in vitro} culturing. With some murine tumors, the loss of surface sialoglycoproteins abolished the ability to transplant tumor cell lines that could previously be transferred within and across strains.\textsuperscript{49-51} Based on literature in murine systems, such changes in surface markers may be overcome by immediate direct, serial transfer of tumor cells between animals, while genetic differences may be circumvented by utilizing conditions favoring tumor growth, such as the dose of cells given to the host animal or the environment into which the cells are injected.\textsuperscript{39,46,48,52-56}

Overall, our results indicate the feasibility of a large animal transplantable tumor model using cells derived from spontaneous or non-genetically induced hematologic malignancies. Further optimization is necessary to achieve tumor growth in host animals without any pre-conditioning or concurrent therapy.
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The authors declare no competing financial interests.

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References


Table 1. Summary of Tumors Identified and Harvested from MHC-Defined Miniature Swine

Table 2. Summary of Established Porcine Tumor Cell Lines. A total of nine porcine tumor cell lines have been established from our MHC defined miniature swine. Of these lines, three are derived from animals that developed spontaneous myeloid leukemias, while the remaining six lines were established from animals with lymphoma or post-transplant lymphoproliferative disease.

Table 3. Summary of In Vivo Tumor Transfer in SCID/NOD Mice and Miniature Swine

Table 4. Summary of Outcomes After In Vivo Transfer of CML-14736 into Miniature Swine

Figure 1. Gross Pathologic and Histologic Findings of Lymphoma (PTLD) in Miniature Swine. Animals that developed lymphomas or PTLD typically had pronounced lymphadenopathy, as represented by animal 17018 (a). Lymph node tissue harvested from these animals demonstrated destruction of normal architecture and predominance of abnormal cells ((b) H&E 40x left, 400x right), represented by animal 13271.

Figure 2. Gross Pathologic and Histologic Findings of Leukemia in Miniature Swine. The most consistent finding of animals with leukemias were enlarged liver and spleen, which on palpation were firm and pale in color with visible lesions, as shown by animal 15549 (a). Bone marrow from these animals were predominantly populated with abnormal cells as represented by tissue from animal 14736 ((b), H&E, 400x).

Figure 3. In vitro growth of tumor cell lines. The nine tumor cell lines display varying patterns of growth and sizes in vitro (40x). Most preferentially grow in clusters, although one line, 14736, grows as a single cell suspension. (a) MML-12933; (b) LCL-13271; (c) ML-13381; (d) CML-14736; (e) CML-15433; (f) LCL-15446; (g) LCL-17016L; (h) LCL-17016P; (i) LCL-17018.

Figure 4. Histologic Findings After in Vivo Transfer of LCL-13271 Tumor Cells in SCID/NOD Mice. LCL-13271 injected IP into SCID/NOD mice with tumor growth at 2 months in primary and secondary recipients. Abdominal tumor mass from SCID/NOD primary (a) and secondary (b) recipients of LCL-13271, 400x, H&E. Histologic findings were similar in morphology as compared to the primary tumor (Figure 2).

Figure 5. Histologic Findings After in Vivo Transfer of CML-14736 Tumor Cells into Miniature Swine. CML-14736 grew after in vivo transfer to histocompatible miniature swine pre-treated with TBI. Tumor growth was found at the subcutaneous injection sites ((a) H&E 400x) and in the lungs after intravenous administration ((b), 200x). Immunohistochemistry of the subcutaneous injection site tissue negative staining for CD3, but positive staining for CD16 and CD172a, which was consistent with the surface phenotype of the primary tumor and cultured cells. ((c) 400x)
Table 1. Summary of Tumors Identified and Harvested from MHC Defined Miniature Swine

<table>
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<tr>
<th>Animal</th>
<th>Haplotype</th>
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<th>Clinical Disease Diagnosis</th>
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<td>13271</td>
<td>AD</td>
<td>3 mos</td>
<td>Lymphoma (PTLD)</td>
<td>B-cell</td>
</tr>
<tr>
<td>13381</td>
<td>DD</td>
<td>39 mos</td>
<td>Leukemia</td>
<td>undifferentiated</td>
</tr>
<tr>
<td>13026</td>
<td>DD</td>
<td>50 mos</td>
<td>Leukemia</td>
<td>myeloid</td>
</tr>
<tr>
<td>14806</td>
<td>AD</td>
<td>7 mos</td>
<td>Lymphoma (PTLD)</td>
<td>B-cell</td>
</tr>
<tr>
<td>15005</td>
<td>CC</td>
<td>3 mos</td>
<td>Lymphoma (PTLD)</td>
<td>B-cell</td>
</tr>
<tr>
<td>15568</td>
<td>AD</td>
<td>3 mos</td>
<td>Lymphoma (PTLD)</td>
<td>B-cell</td>
</tr>
<tr>
<td>15399</td>
<td>CC</td>
<td>5 mos</td>
<td>Lymphoma (PTLD)</td>
<td>B-cell</td>
</tr>
<tr>
<td>15446</td>
<td>CC</td>
<td>6 mos</td>
<td>Lymphoma (PTLD)</td>
<td>B-cell</td>
</tr>
<tr>
<td>15549</td>
<td>HH</td>
<td>30 mos</td>
<td>Leukemia</td>
<td>myeloid</td>
</tr>
<tr>
<td>16556</td>
<td>AD</td>
<td>4 mos</td>
<td>Lymphoma (PTLD)</td>
<td>B-cell</td>
</tr>
<tr>
<td>17016</td>
<td>AD</td>
<td>4 mos</td>
<td>Lymphoma (PTLD)</td>
<td>B-cell</td>
</tr>
<tr>
<td>17018</td>
<td>AD</td>
<td>4 mos</td>
<td>Lymphoma (PTLD)</td>
<td>B-cell</td>
</tr>
<tr>
<td>17102</td>
<td>AD</td>
<td>4 mos</td>
<td>Lymphoma (PTLD)</td>
<td>B-cell</td>
</tr>
</tbody>
</table>

*N/A = diagnosis made based on histologic findings: cellular material not available for phenotyping by FACS
Table 2. Summary of Established Tumor Cell Lines

<table>
<thead>
<tr>
<th>TUMOR LINE</th>
<th>DOUBLING TIME (days)</th>
<th>GROWTH CONSTANT (k)</th>
<th>PERTINENT MARKERS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML-12933</td>
<td>2.36</td>
<td>0.2931</td>
<td>CD16, CD25, CD172a</td>
</tr>
<tr>
<td>LCL-13271</td>
<td>0.984</td>
<td>0.7042</td>
<td>CD2, CD25, anti-mu heavy chain</td>
</tr>
<tr>
<td>UDL-13381</td>
<td>1.65</td>
<td>0.4192</td>
<td>CD25, CD172a, anti-mu heavy chain, anti-kappa light chain</td>
</tr>
<tr>
<td>CML-14736</td>
<td>2.295</td>
<td>0.302</td>
<td>CD9, CD 16, CD25, CD172a</td>
</tr>
<tr>
<td>CML-15433</td>
<td>2.25</td>
<td>0.3078</td>
<td>CD25, CD172a, anti-kappa light chain</td>
</tr>
<tr>
<td>LCL-15446</td>
<td>1.57</td>
<td>0.4416</td>
<td>CD25, CD172a</td>
</tr>
<tr>
<td>LCL-17016L**</td>
<td>1.99</td>
<td>0.3487</td>
<td>CD25, anti-kappa light chain</td>
</tr>
<tr>
<td>LCL-17016P**</td>
<td>0.52</td>
<td>1.324</td>
<td>CD2, CD25, CD172a</td>
</tr>
<tr>
<td>LCL-17018</td>
<td>0.76</td>
<td>0.9128</td>
<td>CD2, CD25, CD172a, anti-kappa light chain</td>
</tr>
</tbody>
</table>

Growth constant (k) = ln 2 / T, where T=doubling time in days

*Note: Full panel includes the following markers - Negative IgG2a, MHC I, MHC II-DR, MHC II-DQ, CD1, CD2, CD3, CD4, CD5, CD8, CD9, CD16, CD21, CD25, CD172a, anti-mu heavy chain, and anti-kappa light chain. All tumor lines were found to be positive for MH

** LCL-17016L and LCL-17016P were both established from the same animal, but from different primary tissues. After growth in vitro, the cells derived from these tissues had different growth rates and surface marker patterns.
Table 3. Summary of *In Vivo* Tumor Transfer in SCID/NOD Mice and in Histocompatible Miniature Swine

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>RECIPIENT ANIMAL SPECIES</th>
<th>OUTCOME</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCL-13271</td>
<td>SCID/NOD mice</td>
<td>successful growth with serial transfer</td>
</tr>
<tr>
<td>MML-12933</td>
<td>SCID/NOD mice</td>
<td>no growth</td>
</tr>
<tr>
<td>CML-14736</td>
<td>histocompatible miniature swine</td>
<td>no growth</td>
</tr>
<tr>
<td>CML-14736</td>
<td>histocompatible miniature swine</td>
<td>successful growth with serial transfer with irradiation pre-treatment</td>
</tr>
</tbody>
</table>
Table 4. Summary of Outcomes After In vivo Transfer of CML-14736 into Histocompatible Miniature Swine

<table>
<thead>
<tr>
<th>ANIMAL</th>
<th>AGE</th>
<th>COANCESTRY</th>
<th>PRE-TREATMENT / IMMUNOSUPPRESSION</th>
<th>TUMOR SOURCE</th>
<th>OUTCOME</th>
</tr>
</thead>
<tbody>
<tr>
<td>16354 (DD2, 10)</td>
<td>3 months</td>
<td>0.94</td>
<td>None / None</td>
<td>Cultured cells – IV, SC</td>
<td>no growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Primary tissue - SC</td>
<td>no growth</td>
</tr>
<tr>
<td>16524 (DD4, G9)</td>
<td>1.5 months</td>
<td>0.87</td>
<td>500cGy TBI / None</td>
<td>Cultured cells – IV, SC</td>
<td>tumor growth at SC sites and in lung</td>
</tr>
<tr>
<td>16523 (DD4, G9)</td>
<td>1.5 months</td>
<td>0.87</td>
<td>300cGy TBI / None</td>
<td>Cultured cells – IV, SC</td>
<td>tumor growth at SC sites only</td>
</tr>
<tr>
<td>16526 (DD4, G9)</td>
<td>3 months</td>
<td>0.87</td>
<td>300cGy TBI / None</td>
<td>Cultured cells - IV, SC</td>
<td>tumor growth at SC sites only</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Passaged cells - SC</td>
<td>tumor growth</td>
</tr>
<tr>
<td>16527 (DD4, G9)</td>
<td>4 months</td>
<td>0.87</td>
<td>100cGy TBI / None</td>
<td>Cultured cells - IV, SC</td>
<td>no growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Passaged cells - SC</td>
<td>no growth</td>
</tr>
<tr>
<td>16525 (DD4, G9)</td>
<td>2 months</td>
<td>0.87</td>
<td>None / CyA (30d)</td>
<td>Cultured cells – IV, SC</td>
<td>no growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Passaged cells - SC</td>
<td>no growth</td>
</tr>
</tbody>
</table>
Figure 1. Gross Pathologic and Histologic Findings of Lymphoma (PTLD) in Miniature Swine
Figure 2. Gross Pathologic and Histologic Findings of Leukemia in Miniature Swine
Figure 3. In vitro growth of tumor cell lines
Figure 4. Histologic Findings After *in Vivo* Transfer of LCL-13271 Tumor Cells in SCID/NOD Mice
Figure 5. Histologic Findings After *in Vivo* Transfer of CML-14736 Tumor Cells into Histocompatible Miniature Swine
Establishment of transplantable porcine tumor cell lines derived from MHC inbred miniature swine

Patricia S Cho, Diana P Lo, Krzysztof J Wikiel, Haley C Rowland, Rebecca C Coburn, Isabel M McMorrow, Jennifer G Goodrich, J Scott Arn, Robert A Billiter, Stuart L Houser, Akira Shimizu, Yong-Guang Yang, David H Sachs and Christene A Huang