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14. ABSTRACT

The lack of faithful animal models limits cancer research. One of the major factors is that regular animal models are a mixture of human tumor cells supported by mouse stroma cells, and this can have a major negative impact in research, particularly that aiming at unraveling the genetic basis of cancer (as this disrupts gene expression), and cancer stem cell and stroma studies. Aims: Generate a fully humanized and individualized model (XactMice) by acquiring and engrafting paired tumor and immortalized bone marrow precursors from the same patient in an immune-deficient mouse model. Our second Aim is to demonstrate the stability and authenticity of XactMice by comparing its gene expression, cancer stem cell profile, and drug sensitivity with a regular xenograft, and the patient's gene profile and outcome. Results: In the first year of funding we have 1) improved the methods for stabilized bone marrow precursor generation, enabling the use of lower amounts of initial patient cells; 2) demonstrated human tumors grow on a humanized mouse model; 3) shown that human stroma homes in to the tumor by dual DNA analyses; and 4) initiated patient material collection and processing. Impact on Cancer Research/ Patients: This project could result in establishing a seemingly ideal model for cancer research, with especial potential to unravel the interactions between tumor and stroma. The potential repercussions in our understanding of processes like angiogenesis and metastasis are profound. This work has the potential to yield high impact data and open new avenues of investigation into mechanisms and targeting of tumor dormancy.

15. SUBJECT TERMS
Humanized animal model, stroma invasion, head and neck cancer

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INTRODUCTION:
The objective of this Department of Defense New Investigator Award for Genetic Research is to develop an ideal animal model of human cancer. We have generated a direct patient tumor model of head and neck squamous cell cancer (HNSCC), but we documented early mouse stroma invasion, and an initial change in gene expression of immune and epithelial genes. A limitation of current in vivo models of cancer is that they end up being a mixture of human tumor cells and mouse stroma cells providing support (new vessel formation, architectural structure). This can impact the validity of the models, may be responsible for their poor predictive value, and may be a major roadblock for key research areas. We have hypothesized that 1) it is feasible to reconstitute a mouse model with both tumor and hematopoietic stem cells (HSC) from the same patient; and 2) this fully humanized and homologous cancer model will faithfully replicate the originator tumor. We have structured our plan in two Aims: 1) Generate XactMice by acquiring and engrafting paired tumor and immortalized bone marrow precursors from the same patient in an immune-deficient mouse model; and 2) Demonstrate the stability and authenticity of XactMice by comparing its gene expression, cancer stem cell profile, and drug sensitivity with a regular xenograft, and the patient's profiles and outcome. This Department of Defense New Investigator Award for Genetic Research grant proposal represents a seamless integration of expertise at the basic, translational and clinical research science levels. Thus this Project will help foster the kind of collaboration that is so critical to make quantum leaps in research. The area where such an ideal model can have a largest impact is genetic research, as it will enable better correlating mutational alterations with their true impact in gene and protein expression in a fully humanized environment. This will in turn allow more accurate target identification, and therefore faster drug development. In second place, this approach will yield a system ideally suited to study tumor initiating (stem) cells and/or stroma cell dynamics in head and neck cancer. This will enable an unprecedented insight into the processes of tumor growth and metastatsis. If positive, this project will constitute a landmark in cancer research, and has the potential to be paradigm-changing.

BODY:
STATEMENT OF WORK AND ACCOMPLISHMENTS

Because tasks 1-2 span over the first two years of the award and tasks 3-7 spam over the entire award progress will be referred to as accomplishment of the portion corresponding to each specific year.

Task 1 – Obtain fresh head and neck squamous cancer fresh tissue from patients undergoing surgery to generate initial implanted tumors. Months 1-24.

a. Regulatory issues – Approval from the Colorado Multiple Institutional Review Board (protocol 08-0552) was obtained on 7/22/2008 to collect the required samples from the University of Colorado Head and Neck Cancer clinic.
b. Routine implantation of cases is ongoing and will continue through several years to build a regular, nude mice-based colony.
c. We have already implanted 25 cases of head and neck cancer, as noted in the preliminary data results, of which the majority are growing after successful engraftment.
d. Antitumor experiments are ongoing in such cases and we have acquired the drugs and developed the administration protocols that mimic what the patients receive in the clinic.

Task 1 progress in first year of funding. Task is ongoing as planned, and we have completed the objectives of year 1 (**items a-d**). Since initiating the award the protocol was adapted and approved by COMIRB to enable all

sample collections. Currently we have implanted 44 samples from patients, which indicates a progress of almost 2 patients per month. We have been successful in collecting paired tumors simultaneously implanted from primary and node metastasis. This opens the possibility, once the full technology is available and if those patients give rise to stabilized Lt-HSC lines, to study determinants of metastasis in XactMice, and how a humanized stroma influences. **Figure 1** shows example of cases implanted with different degrees of differentiation, and **Table 1** summarizes the status of the baseline colony by implantation characteristics.

During the **second year of the award** we expect to continue enrolling and engrafting patients, and we anticipate no delays in completing this task.

Task 2 – Obtain linked clinical data from the patients from Task 1 and determine eligibility based on the type of therapy they receive after initial surgical diagnosis
Months 1-24

a. Clinical abstraction of the electronic and paper chart data for local cases has begun and will continue through the first two years of the project, reflecting the length of time required to fully obtain the needed clinical data for linkage and outcomes analysis with the therapeutic results.
b. Determining which patients are scheduled to receive induction chemotherapy and coordinate the timing of their clinical care to the acquisition of the blood samples on days 7-10 of their cycles.
c. Determine whether a blood extraction has been successful or not in order to schedule a re-draw on a subsequent cycle if possible.

Task 2 progress in first year of funding. Task is ongoing as planned, and we have completed the objectives of year 1 (**items a-c**). We have developed a secured, on-line database tool to include, access and analyze clinical from patients. This is enabling us to get data in real-time which allows to predict when patients are coming to clinic and when they are receiving therapies that will enable successful blood and Lt-HSC acquisition.

During the **second year of the award** we expect to continue inputting data from patients and adding functionalities to the database, and we anticipate no delays in completing this task.

Task 3 – Acquire peripheral long-term hematopoietic stem cells (Lt-HSC) while patients receive induction chemotherapy
Months 3-27

a. We will acquire samples from 15-20 patients over the three-year duration of this project.
b. Collection of peripheral blood of patients treated with G-CSF. Peripheral blood (10 ml) will be obtained from these HNC patients 3-7 days after G-CSF treatment, when the circulating Lt-HSCs are increased from 1-2/mL to 10-20/mL. We will need 10 ml of peripheral blood from those patients in order to generate. All patient recruitment and handling of donated blood will be carried out in accordance to the University of Colorado Denver Institutional Review Board COMIRB 08-0552 approved protocol.
c. We will isolate Lt-HSCs with magnetic beads coated with human CD34 antibody and culture cells in 96 well plates in HSC media on a feeder layer of mitomycin-C treated whole cord blood cells.

Task 3 progress in first year of funding. Task is ongoing as planned, and we have completed the objectives for year 1 (**items a-c**). Despite substantial challenges in measuring and isolating Lt-HSCs from small amounts of human peripheral blood we have three patients at different stages of identification/isolation/stabilization of precursors. These include a case of a tonsil cancer patient where we were able to implant separately the primary tumor and a lymph node metastasis (CUHN047-P, CUHN047-N). If we are successful in establishing XactMice on this case we will have a phenomenal tool to study the differences between primary tumors and metastases and how the stroma is involved. During the first 6-9 months of the award we have also focused in developing the assays to accomplish Task 3-7 in a human setting.

During the **second year of the award** we expect to continue identifying/acquiring samples from patients on treatment.

Task 4 – Generation of conditionally immortalized long-term hematopoietic stem cells (Ctlt-HSC)
Months 3-27

a. Immortalization of Lt-HSCs with Tat-MYC and Tat-Bcl-2. HIV-1 Tat fusion proteins (Tat-*MYC* and Tat-Bcl-2).
b. We will culture the mixed cell population in human stem cell media (Stemline II), supplemented with recombinant human IL-3, IL-6 and stem cell factor.
c. We will add recombinant Tat-MYC and Tat-Bcl-2 proteins that were produced under low endotoxin conditions. The cells will be incubated in 24 well plates in 1 ml of medium, with a starting density of 2x106 cells per well. The medium will be replaced every 2 days and the cultures will be carried at least 21 days.
d. We will ascertain expansion of the HSC population by flow cytometric staining for CD34, CD133, CD48/SLAM, CD150/SLAM and lineage markers (CD3, CD19, Mac-1, ter-119, and Gr-1), in combination with cell counts.
e. The analysis will enable us to determine whether this approach is favoring the specific expansion of the Lt-HSC fraction (CD34+, lin-), as we have observed previously with retroviral transduction of Bcl-2 and MYC.ER.
f. The expanded cell population will be cryopreserved after characterization of surface marker expression by flow cytometry.

Task 4 progress in first year of funding. Task is ongoing as planned, and we have completed the objectives for year 1 (**items a-f**). We have made progress on the generation of LT-HSC cell lines in the absence of genetic modification, and have improved the methods for stabilized bone marrow precursor generation, enabling the use of lower amounts of initial patient cells. While the general methodology is similar to what was presented in the Preliminary Data, we have improved even further the method for the production of recombinant purified HIV-1 Tat fusion proteins (Tat-MYC and Tat-Bcl-2), and as such a summary of those improvements is presented here (**Figure 2**). Those fusion proteins were then purified to homogeneity and low endotoxin conditions, while retaining their activity in a biological assay (rescuing the survival of activated T and B cells following withdrawal of cytokines; **Figure 3**). We then deployed the recombinant fusion proteins in the context of conditional immortalization of primary human Lt-HSCs (**Figures 4 & 5**). Those cell lines present with the surface phenotype of a long-term HSC, and were able to give rise to human lymphoid cells in xenochimaeric mice. These technical steps are critical for the generation of Ctlt-HSC cell lines from HNC patients.

Despite substantial challenges in measuring and isolating Lt-HSc from small amounts of human peripheral blood we have three patients at different stages of identification/isolation/stabilization of precursors. These include a case of a tonsil cancer patient where we were able to implant separately the primary tumor and a lymph node metastasis (CUHN047-P, CUHN047-N). If we are successful in establishing XactMice on this case we will have a phenomenal tool to study the differences between primary tumors and metastases and how the stroma is involved. During the first 6-9 months of the award we have also focused in developing the assays to accomplish Task 3-7 in a human setting.

During the **second year of funding** we expect to continue identifying/acquiring samples from patients on treatment.

Task 5 – Conditioning and engraftment of human hematopoietic precursors on mice
Months 3-27

a. An IACUC approved animal protocol is already in place that covers the research outlined in this task. All animal studies are performed in full IACCUC compliance.
b. Cohorts of NOD/SCID/γc-/- mice that will be sublethally irradiated (300 Rads). These models of xenotransplantation have been previously described and shown to effectively allow for the development of human hematopoietic lineages upon transplantation of human HSCs.
c. Ctlt-HSC will be injected IV 7 days after irradiation.

d. We will maintain the transplanted mice on Septra and bleed them every four weeks after transplantation in order to monitor for the presence of GFP+ cells in the blood as well as specific lineage markers for T and B-cells. Initially, we will tail-bleed the bone marrow transplant recipient mice and stain their peripheral blood leukocytes with antibodies for human B-cell, T-cell and myeloid cell markers.
e. The total number of red and white blood cells will be determined with a Hemavet apparatus, that is available for our use at the University of Colorado Cancer Center. These data will allow us to determine whether the HSCs used for the transplant were able to successfully engraft and give rise to a phenotypically normal hematopoietic compartment. This will be the first measure of appropriate homing and successful engraftment.
f. We plan to begin to functionally characterize their hematopoietic compartment 12 weeks after transplantation. Once engraftment is verified and we obtain confirmation of a human phenotype we will initiate the process of tumor implantation.
g. We expect we will attempt 15-20 immortalizations of Lt-HSC to obtain 12-15 successful ctlt-HSC that will be necessary to obtain 8-10 successful tumor (thus dual) engraftments.
h. We expect we will implant 3-4 irradiated NOD/SCID/$gc^{-/-}$ mice for each of 15-20 cases were we are able to generate ctlt-HSC. We estimate 60 mice will be used.

Task 5 progress in first year of funding. This task is being accomplished as planned (pertaining **items a-f**). In summary we have been successful in engrafting human Ctlt-HSC to give rise to scaled-up batches of mice to develop the assays needed to conduct and evaluate the full experiments.

During the first 6-9 months of the award we have focused in developing the assays to accomplish Task 3-7 in a human setting. To this end, and in order not to have to wait for a successful paired Lt-HSC and Ctlt-HSC, we have constructed two cases of XactMice using donor human cord blood progenitors (**Figure 6**). This is critical as up until now we had given rise to individual mice, but not cohorts, and we needed to develop the procedures to scale-up mice generation. We have been successful in generating 2 separate cohorts of >10 XactMice each, with several other batches being generated.

Subsequently we have implanted early passage (F2) tumor from two cases CUHN004 and CUHN013 in nu/nu, NSG and XactMice (approximately 5 nu/nu, 5 NSG, 5 XactMice), and have been able to study their dynamic growth (**Figure 7**). We have repeated these experiments twice in two successive cohorts.

Generating these XactMice using donor human cord blood progenitors was a needed step in order to unequivocally prove progenitor homing from the humanized bone marrow to the tumor. This served two additional purposes: 1- we were able to perfect the assays so by the time the dual individualized XactMice they are ready; and 2- the dual origin of the DNA enables differentiating it and unequivocally determining whether human stroma re-located from the bone marrow to the tumor. Because their origin is human its DNA gets amplified in fingerprinting assays, but because it is not from the same origin than the tumor DNA; thus, if we identified 2 sources of human DNA from a single tumor growing on XactMice (but not nu/nu or NSG) it would provide 100% proof that the second DNA came from stromal/precursor cells from the bone marrow (see below for results).

During the **second year of funding** we expect to initiate transduction from successful Ctlt-HSC from the three patients that are at different stages of identification/isolation/stabilization of precursors, plus those subsequent patients that will be identified. We will implement the embellishment to the techniques developed using the donor progenitor humanization process.

Task 6 - Generation and treatment of cohorts of mice-bearing tumors
Month 6-30

a. An IACUC approved animal protocol is already in place that covers the research outlined in this task. All animal studies are performed in full IACCUC compliance.

b. When engraftment of the ctlt-HSC is verified and at least 2-3 mice are available, we will go to the main DTPM colony and evaluate growth of the parent tumor. This will occur for each of the 15 cases we expect that will engraft.
c. If growth is successful, we will divert part of that generation (be it F1 or already F2) outside of the bank. Tumors will be resected and evenly distributed into the humanized NOD/SCID/γc-/- mice and regular NOD/SCID/γc-/-mice, on groups of similar size. If tumor is scarce, 2-3 mice per type will be implanted. If tumor is abundant, we will attempt implantation of as large a colony is possible, with the limitation of the availability of humanized mice.
d. We will test cetuximab, docetaxel and the combination in 8-10 cases. When tumors reach the target volume we will distribute them in 4 groups (at least n=10 tumors per group): control, cetuximab 40 mg/kg 2/week IP, docetaxel 30 mg/kg 1/week IP, or cetuximab 40 mg/kg 2/week IP plus docetaxel 30 mg/kg 1/week IP. Treatment is planned to last 4 weeks. Tumor size will be evaluated 2/week using the formula: volume = [length x width2]/2. At the end of the experiment, tumors will be extracted and flash-frozen (n=4; one half), embedded in paraffin (n=4; second half), and prepared fresh for FACS counting and sorting (n=3-4, see below methods).
e. For each efficacy study we will utilize (on average) 40 mice per case. This is because we will implant 20 conditioned ctlt-HSC-bearing NOD/SCID/$gc^{-/-}$ mice (40 tumors) and 20 regular NOD/SCID/$gc^{-/-}$ mice (40 tumors) with tumors, in order to obtain sufficient tumors for the efficacy studies in each group. The estimated number of mice is 300-400.

Task 6 progress in first year of funding. The assays required to accomplish this task are being developed as planned (**items a-c**). We have been successful in generating initial test cohorts and then implanting tumors, and have been able to compare their dynamics for growth and to do time-course experiments comparing the groups. We have not yet initiated treatment of XactMice cohorts with antitumor agents, as we have not yet generated cohorts of the appropriate size (although we have initiated scaled up stroma and biomarker testing as a prelude to the therapeutic experiments). Three patients are at different stages of identification/isolation/stabilization of precursors, prior to XactMice implantation. **During year 2 of this award** we expect to initiate large cohort therapeutic testing now that post-treatment evaluation assays are developed.

Task 7 - Generation and analysis of flow cytometry, gene expression and IHC data between the originator patient tumor, and both sets of mice-generated tumors, and comparison to clinical data set with outcomes analysis
Month 6-27 for molecular data generation work
Months 12-36 for molecular data analysis and interpretation work

a. The identification of CSC will be done utilizing the stem cell markers CD24, CD44, and aldehyde dehydrogenase 1 (ALDH).
b. Cells will be sorted with a Beckman Coulter MoFlo cell sorter equipped with a iCyt Lyt 200S 488nm laser and a Melles Griot HeNe 633nm laser.
c. Cells will be gated using linear forward scatter and linear side scatter and doublets will be excluded using a forward scatter height by area plot.
d. Gene mutation testing. The Illumina Genome Analyzer identifies DNA mutations (including, SNPs, insertion and deltion, and copy number variations. In-house bioinformatics software will be used to identify these mutations.
e. Gene expression in the original and subsequent passes will be profiled using the Affymetrix GeneChip® Human Gene 1.0 ST array that has 764,885 probes covering 28,869 human genes. Data extraction will be done with Affymietrix GCOS software. Data normalization will be processed using Robust Multiarray Average (RMA) algorithm implemented in R Bioconductor.
f. Immunohistochemical (IHC) analysis will be performed on 99-core, 1.4mm-diameter tissue micro arrays (TMA) made of surgical and biopsy specimen blocks
g. TMAs will be constructed using a manual Tissue Puncher/Arrayer.
h. We will generate 3-4 TMA blocks with the estimated 10 patients and samples from both XactMice and regular mice tumors. Each complete patient set will be composed of 32 cores (2 cores from baseline sample, 2 normal mucosa cores, 2 cores from each F1, F2 and F3 passage in

XactMice and regular mice, and 2 cores from each treatment group [estimated as 4 groups] in XactMice and regular mice).

i. The markers we plan in principle to analyze include CD24, CD44, ALDH1, and CD133. In pre- and post-treatment samples we will also study the EGFR pathway (EGFR, perk) in endpoints we have already developed and tested previously. For IHC staining we will follow the methodology developed by us for the markers of interest.
j. Briefly, TMA slides will be de-paraffinized and re-hydrated in graded concentrations of alcohol by standard techniques before antigen retrieval in citrate buffer pH 6.0 for 20 minutes. Next, the slides will be cooled for 20 minutes before washing in 1x TBST.
k. Slides will be incubated in 3% H2O2 for 10 minutes, followed by the appropriate dilution of primary antibodies (CD24, CD44, and ALDH) for 60 minutes.
l. Staining will be developed using the DAKO LSAB+ System: biotinylated link for 10 minutes, streptavidin for 10 min, and substrate-chromagen Solution for 5 minutes. The intensity (0, 1+, 2+, 3+) and the percentage (0% - 100%) of cells positive will be considered. For statistical analyses, an index of intensity x percentage will be calculated.
m. With the results of the therapy we will conduct a correlation analysis with CSC subpopulations..
n. Flow cytometry, gene expression, and IHC data will be linked to outcomes data, and relationships statistically evaluated.
o. Results of the analysis will be returned to Dr. Jimeno for full interpretation and formal data preparation for publication.
p. Manuscripts will be written and submitted for publication.

Task 7 progress in first year of funding. The assays required to accomplish this task (pertaining **items a-m**) are being developed as planned. We have been successful in generating initial test XactMice cohorts and then implanting tumors, and have been able to compare their dynamics for growth and to conduct time-course experiments comparing the different groups. We have been particularly successful in developing assays for cancer stem cell (CSC) identification as planned using CD24, CD44, and ALDH, as well as human stroma characterization with the most selective marker being CD151.

We compared the CSC composition of tumors by conducting flow cytometry of grown tumors and to compare across groups. In **Figure 8** we compare the CSC marker CD44 profiles between nu/nu, NSG and XactMice tumors.

As introduced above, in order to unequivocally prove progenitor homing from the humanized bone marrow to the tumor, we constructed two cases of XactMice using donor human cord blood progenitors, and engrafted 2 different strains of tumor (one from a male patient, one from a female patient) on two cohorts of nu/nu, NSG and XactMice. Because the origin of the stromal cells is human its DNA gets amplified in human DNA fingerprinting assays, but because it is not from the same origin than the tumor DNA, if we identified 2 sources of human DNA from a simgle tumor growing on XactMice (but not nu/nu or NSG) it would provide 100% proof that the second DNA came from stromal/precursor cells from the bone marrow.

It was critical to determine whether stroma was actually homing in to the tumors once implanted. This had not been initially identified as a study priority, but later experiments indicated that this corresponds to 1- a valid surrogate to gauge the success of the experiment and 2- an area of intrinsic potential research value. In **Figure 9** we can see how XactMice had 3-fold increase in human stroma by CD151 compared to nu/nu and NSG-originated tumors after a 3-month period of growth. This is particularly striking considering tumors increased in volume from the 1-month to the 3-month timepoint by at least 3-fold, so the absolute increase in CD151+ cells was 10-fold in XactMice overall. Provided these cells are truly stromal cells, they or their precursors may have originated in the humanized immune systems of the XactMice. This model can provide valuable information about the composition of the stroma in human cancer xenografts and help elucidate the role of the stroma in cancer growth and metastasis.

We estimated the presence of mouse and human stroma utilizing a differential fluorescence in situ hybridization (FISH) DNA staining assay, where human DNA stains as red and mouse DNA stains as green, providing semi-quantitative assessment of the ration human/mouse cells (**Figure 10**). By pathologic analyses in XactMice there was less mouse stroma compared to NSG mice in the analyzed cases.

To unequivocally prove that there was bone marrow-originated stroma in the tumors we designed a DNA fingerprinting assay to confirm our observation. The origin of the CD151+ cells collected by cell sorting was determined by STR analysis, using primer sets widely employed for this purpose. The STR fingerprint obtained in this analysis was compared to that obtained by amplification of previously isolated mouse and genomic DNA from the patient tumor that originated the xenograft (CUHN013) (**Figure 11**). Since unique PCR banding patterns observed from XactMice CD151+ cells are not present in the human DNA from the tumor cells, it can be hypothesized that these cells had been incorporated into the tumor stroma after having originated in the XactMice's humanized bone marrow. Work is ongoing to conduct gene expression analyses to compare the three origins of the tumor.

We have not yet initiated treatment of XactMice cohorts with antitumor agents, as we have not yet generated cohorts of the appropriate size (although we have initiated scaled up stroma and biomarker testing as a prelude to the therapeutic experiments). Three patients are at different stages of identification/isolation/stabilization of precursors. **During year 2 of this award** we expect to initiate large cohort therapeutic testing now that post-treatment evaluation assays are developed.

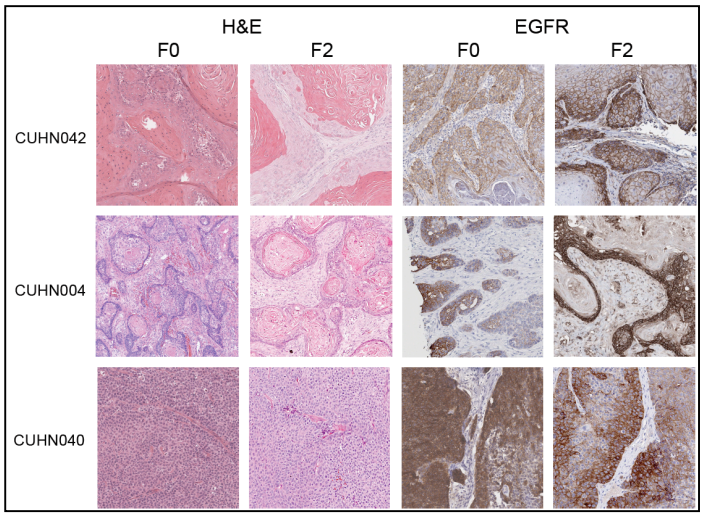


Figure 1. Examples of tumors from standard (nu/nu) implantation model (F2), showing the original patient tumor (F0) as reference.

Table 1	Number (%)	Growing (%)
Cases		
Consented	51 (100)	25 (51)
Implanted	50 (98)	25 (50)
Site		
Oral and tonsil	27 (54)	13 (51)
Pharynx	3 (7)	1 (33)
Larynx	10 (25)	5 (50)
Other & Skin	10 (20)	6 (60)
Cases		
Primary	24 (48)	12 (50)
Relapses	26 (52)	13 (50)
Cases		
F1 < 6 months	12 (24)	3 (25)
F1 > 6 months	38 (24)	22 (58)

Table 1. Patient characteristics and implantation success by subgroup. Notable the engraftment rate for the different subgroups is similar so the resulting platform is indeed representative of the patient population.

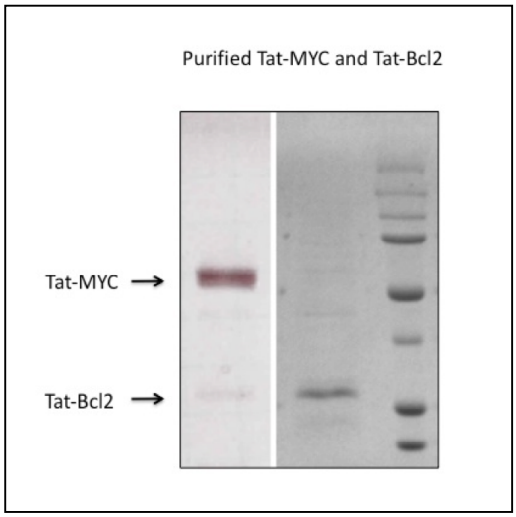
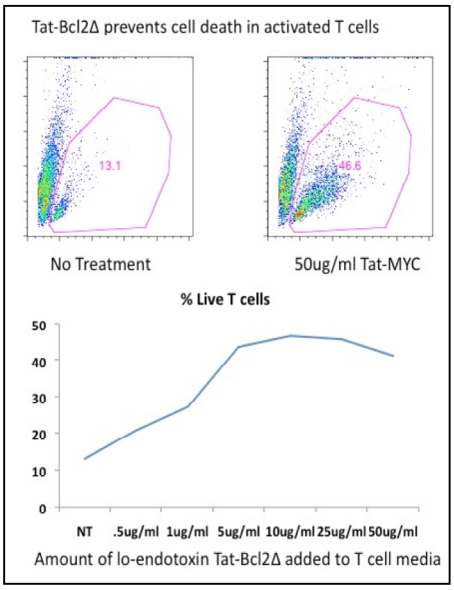
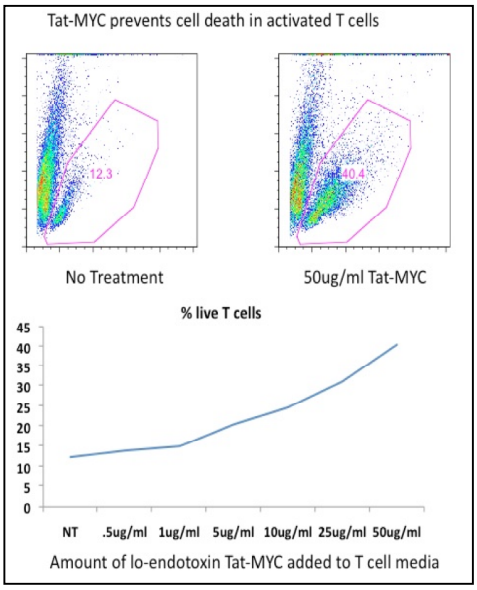


Figure 2. Silver Staining of recombinant purified Tat-MYC and Tat-Bcl-2 fusion proteins. We constructed and cloned into a bacterial expression plasmid. The plasmid was transduced into E. Colli that were then grown and induced with IPTG. The cells were lysed and lysates were clarified and run. The relevant fractions were then run on a sizing column. The relevant fractions were pooled and used for further endotoxin removal. A sample of the protein was run on a 15% SDS-PAGE gel and stained with a silver stain.

Figure 3. Purified Tat-Bcl-2 and Tat-MYC proteins are biologically active. Murine CD4+ T cells were purified and activated with antibodies to CD3 and CD28 for 72 hours. The cells were then washed and live cells were enriched on a ficoll-hypaque gradient. Live cells were then incubated in media alone, or media supplemented with Tat-Bcl-2 and TAT-Myc. The number of live cells was determined by FACS through gating on the live cell population by Forward and Side scatter (top two panels), as well as staining for live cells by exclusion of 7AAD.





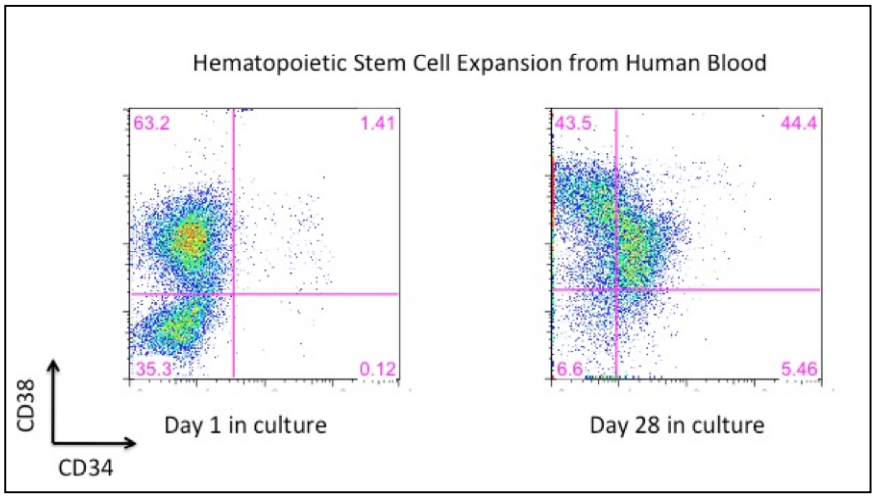
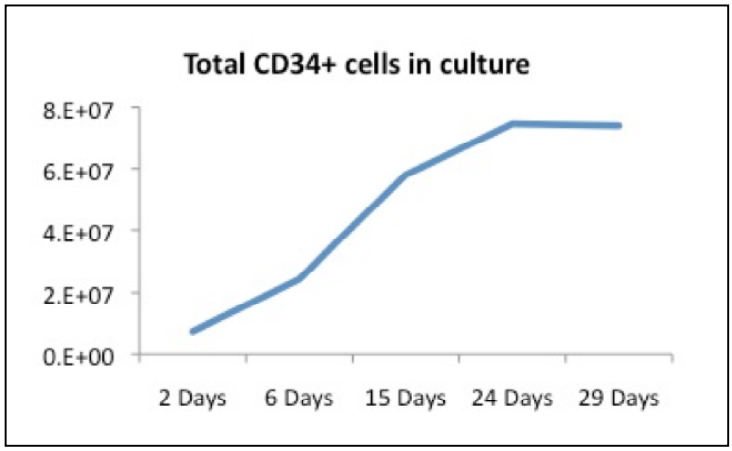


Figure 4. Expansion of primary human Lt-HSCs in vitro by culturing with Tat-MYC and Tat-Bcl-2. We obtained primary human cord blood cells, and lysed the red blood cells by incubation in ahypotonic lysis buffer. The remaining cells were then cultured in stem span media (Stem Cell Technologies, Vancouver Canada), supplemented with standard stem cell cytokines as well as Tat-MYC and Tat-Bcl-2. 14 days later, we observed an increase in the frequency of human Lt-HSCs from a starting frequency of 1.5% to 44.4%.

Figure 5. Magnitude of expansion in vitro of human LT-HSCs with the recombinant protein approach. We stained samples obtained from cultures set up as described in Figure 4, and analyzed them by FACS for the presence of human Lt-HSCs. The diagram represents the total number of LT-HSCs in the cultures. Importantly, the total number of human LT-HSCs is increasing steadily in the cultures over the period in which they are analyzed. The human HSCs obtained from this culture on day28 were used to reconstitute the mice discussed in Figure 6.



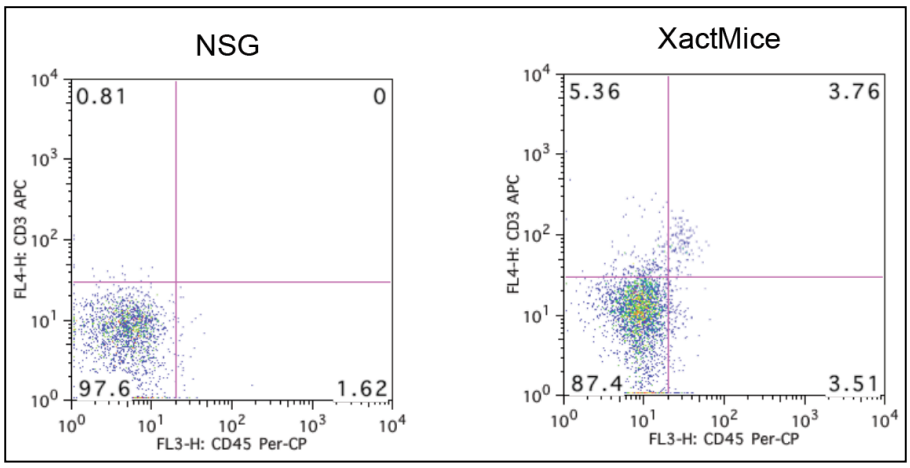


Figure 6. Flow cytometry to assess mouse blood for human CD45+ and CD3+ cells. Peripheral mature human precursors are detected in XactMice after successful Ctlt-HSC implantation. Shown here is one of the 12 transduced mice, with successful engraftment observed in 10.

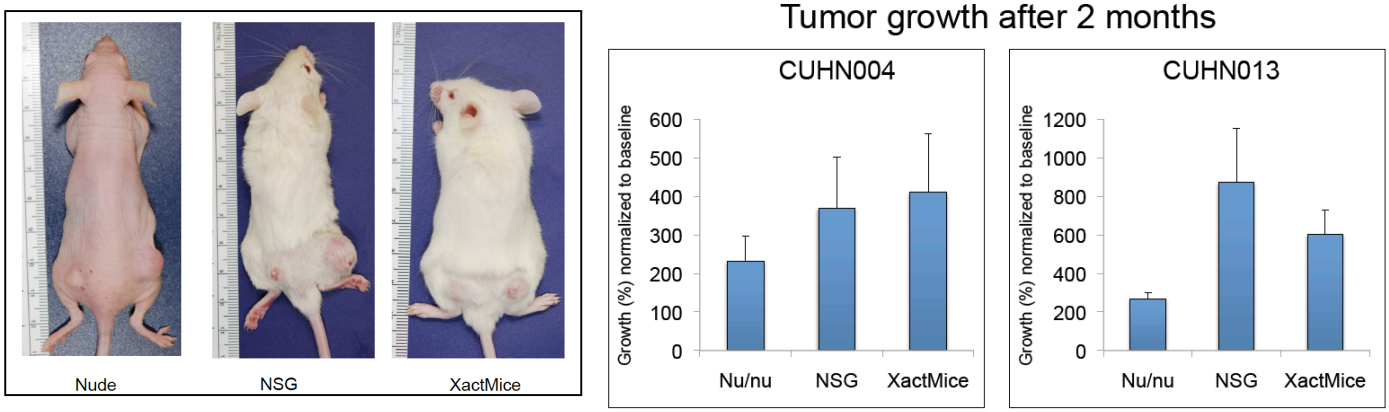
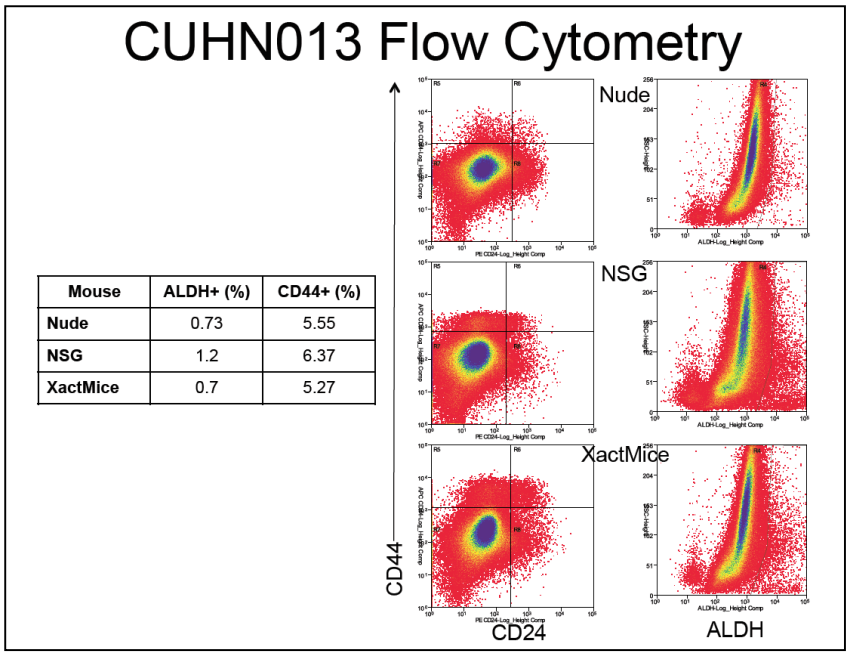


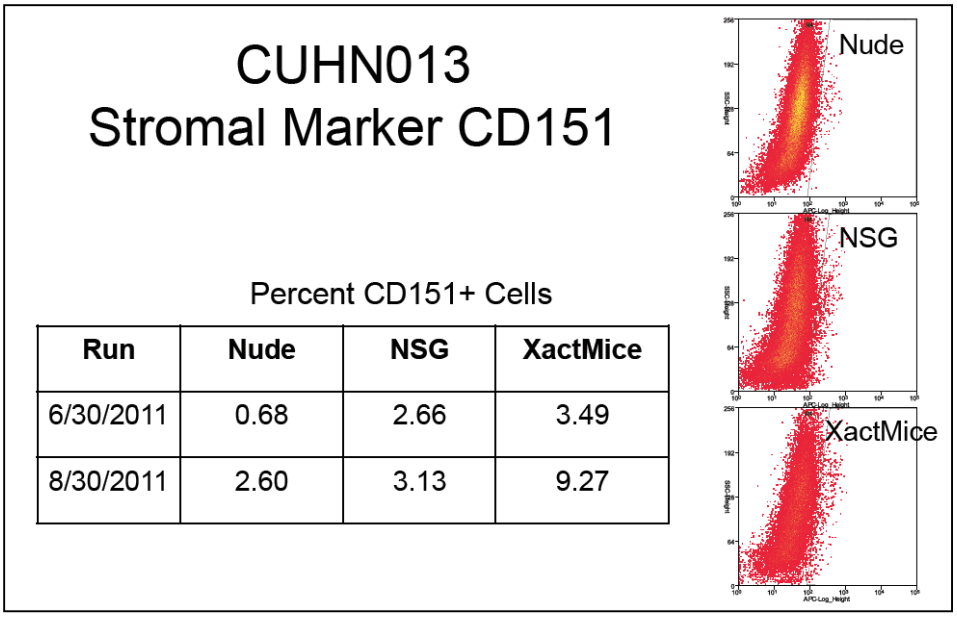
Figure 7. A. Tumors growing on nu/nu, NSG and XactMice models. B. Growth dynamics by subgroup. No definitive trend was observed in the growth of the three types, in either of the cases engrafted. Data shown are averages of two subsequent experiments.



Mouse	ALDH+ (%)	CD44+ (%)
Nude	0.73	5.55
NSG	1.2	6.37
XactMice	0.7	5.27

Figure 8. CSC comparison between the three cohorts of mice. The profile in the CSC markers CD24, CD44 and in the positivity of ALDH showed that XactMice tumors resembled more closely those from nu/nu mice than those from NSG mice. However there were no significant differences between the groups.

Figure 9. Stroma comparison. We tested a series of stroma markers to identify which would enable a more accurate identification of human stromal elements. CD151 (also known as tetraspanin cell surface protein) was measured in tumors 1 and 3 months after implantation. Whereas in the early time-point XactMice showed a higher percentage of human stroma within the tumor, it was after 3 months where the differences seemed to be clearer. In this setting XactMice had 3-fold increase in human stroma by CD151 compared to nu/nu and NSG-originated tumors.



Run	Nude	NSG	XactMice
6/30/2011	0.68	2.66	3.49
8/30/2011	2.60	3.13	9.27

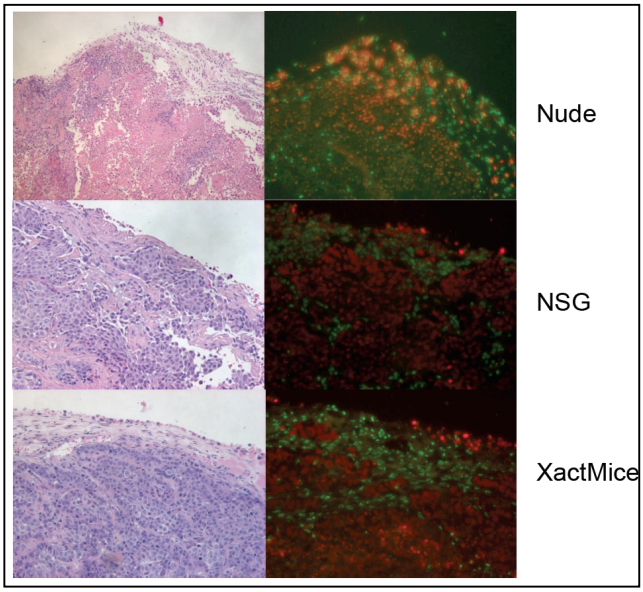


Figure 10. Cot assay comparison between Nude, NSG and XactMice CUHN013 tumors. Red and green indicate human and mouse DNA, respectively. Although difficult to appreciate in the detail microphotograph, when entire tumors were assessed stroma thickness was diminished in the XactMice tumors compared with the NSG tumors, with a higher red/green ratio. This is suggestive of presence of human stroma elements.

Lane		Band size (bp)	Band size (bp)
L	Ladder		
1.	Mouse tissue	231, 246	162
2.	Patient tumor tissue	234, 248	165
3.	XactMice intratumor CD151+	238	151, 171

Figure 11. DNA fingerprinting assay. In this test we aimed at determining whether stroma cells (CD151+) home into the tumor. We used a XactMice engrafted with human donor-originated Ctlt-HSC and not from the tumor-bearing patient, in order to unequivocally identify the origin of the human cells populating the tumor. The STR fingerprint obtained by amplification of previously isolated mouse (lane 1) and genomic DNA from the patient tumor that originated the CUHN013 xenograft (lane 2) was compared to that obtained from CD151+ cells isolated from XactMice tumors from CUHN013 (lane 3). We utilized two different STR (TPOX and vWA) for completeness. Since unique PCR banding patterns observed from CD151+ cells originiated from XactMice CUHN013 tumor are not present in the human DNA from the original CUHN013 tumor cells, it can be hypothesized that these human cells had been incorporated into the tumor stroma after having originated in the XactMice's humanized bone marrow.

KEY RESEARCH ACCOMPLISHMENTS:

1. Improved the methods for stabilized bone marrow precursor generation, enabling the use of lower amounts of initial patient cells.
2. Identified and collected blood and tumor from prospective patients, and initiated the process of establishing Ctlt-HSC.
3. Were able to implant cohorts of regular and humanized mice (XacMice) with human tumors, demonstrating growth.
4. Documented differences in stroma distribution and lower mouse stroma homing into tumors growing on XactMice.
5. Identified CD151 as a human stromal marker valid for identification and quantitation.
6. Demonstrated the homing of human stromal cells from the bone marrow to the tumor.
7. Characterized the flow of human stromal cells from the bone marrow to the tumor.
8. These results provide insight into fundamental processes related to the relationship between tumor cells and the microenvironment.
9. These results could have implications as to our understanding of tumor metastasis processes and provide a unique tool to identify critical aspects of it.

REPORTABLE OUTCOMES:

National Presentations: none at present.

Manuscripts: none at present.

Abstracts: none at present.

Awards: none at present

Employment or research opportunities:

1. Post-doctoral training for J. Jason Morton PhD, whose position is partially funded by this award.
2. Post-doctoral training for Gregory Bird PhD, whose position is partially funded by this award.

Informatic data bases: none at present, but humanized and non-humanized tumor gene expression profiles will be publically available at time of publication.

Funding applied for based on this work: none at present.

CONCLUSIONS: Our studies thus far have shown that it is feasible to engraft human tumors on humanized mice. This leads to changes in stroma re-population and we have demonstrated human stroma elements homing in from the bone marrow, utilizing a humanized but not fully individualized approach. Understanding how stroma migrates and how this contributes to tumor growth in a fully humanized and fully individualized setting could have array of implications for studying human cancer progression and response to chemotherapy, as well as give an insight in processes such as metastasis.

REFERENCES: none at present.