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14. ABSTRACT

The mammary gland is made up of two distinct epithelial layers that are derived from a common progenitor. Theexperiments in this proposal investigate the processes of mammary epithelial cell differentiation during developmentand tumorigenesis. Using FACS, mammary epithelial cell (MEC) populations from tumors and wildtype tissue wereinvestigated for their outgrowth potential or tumorigenic capacity. We also developed new mouse models and imagingtechniques that can be used to identify viable long-surviving cell populations and characterize these cells in real-timein vivo. We are also interested in studying how genes expressed in different mammary epithelial cell populations affectdifferentiation and tumorigenesis. For these experiments we have developed techniques for viral transduction andtransplantation of primary MECs.

15. SUBJECT TERMS
mammary, stem cell, lentivirus, FACS, cancer, imaging

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INTRODUCTION:

The mammary gland is made up of several epithelial cell populations including luminal and myoepithelial cells that are derived from a common progenitor. The experiments in this proposal investigate the process of mammary gland differentiation during normal growth and tumorigenesis. Using FACS, mammary epithelial cell (MEC) populations from tumors and wildtype tissue was investigated for their outgrowth potential or tumorigenic capacity. We also developed new mouse models that can be used to isolate viable long-surviving cell populations. Once cell populations with slow turnover have been identified they will be characterized using real-time *in vivo* imaging techniques and isolated by FACS for characterization of their outgrowth capacity and gene expression profiles. We are also interested in studying how genes expressed in different mammary epithelial cell populations affect differentiation and tumorigenesis. For these experiments we are developing techniques for viral transduction and transplantation of primary MECs.

Task 1. Characterize mammary epithelial cell populations in tumors and the normal gland. **Complete**

-No new progress has been made toward this aim in the past year.

Summary of Project to Date:

This task seeks to characterize the Sca-1 marker and identify novel markers in the normal mammary gland and tumors that can be used to characterize mammary epithelial cell progenitors during tumorigenic progression. We have found that several mouse mammary tumor models can be divided into "progenitor" and "luminal" phenotypes. Progenitor tumor phenotypes contain cells that express myoepithelial and luminal cell types and often exhibit some degree of cellular organization upon histological analysis. In many of these tumors the K14 (myoepithelial) and K8 (luminal) positive cells are structured into distinct concentric layers suggesting organizational signals are maintained. Putative progenitor cell markers such as keratin-6 and Sca-1 are also highly expressed in these tumors and their initial hyperplasias. Interestingly, progenitor type tumors are mainly observed from oncogenes in the Wnt/β-catenin pathway including Wnt-1, Myc, and β-catenin. Furthemore, squamous metaplasia is often observed in these tumors. In contrast, luminal tumor phenotypes are mainly observed with receptor tyrosine kinase pathway oncogenes such as Neu, Ras and PyMT. These tumors are poorly organized and express almost exclusively the K8 luminal epithelial marker. These data suggest that specific oncogenic pathways expand a particular cell type that can give rise to the tumor mass. Since tumors result from clonal expansion the "progenitor tumor" phenotype must be derived from a cell type that can give rise to both luminal and myopeptithelial cells while "luminal tumors" are somehow restricted to a luminal-type differentiation. Further studies will determine whether progenitor type hyperplasias have enhanced stem cell activity when assayed by limited dilution transplantation.

We have also identified a new marker, CD24, expressed on a subset of Sca-1 MECs. We have further characterized cells that express this marker in Task1. We have examined the expression of CD24 and Sca-1 in mouse mammary tumor models and we have performed transplantation and IHC on sorted Sca-1/ CD24 cell populations. Several

observations and technical issues have been raised from these experiments that must be addressed in further experiments. For example, the expression of Sca-1 increases from about 30% positive total cells in freshly prepared primary MECs to about 80% in MECs cultured on plastic. Furthermore, many immortalized MEC cell lines such as EpH4 and SCP2 cells also express high levels of Sca-1. However, cells that are cultured in Matrigel express Sca-1 in only about 15-20% of epithelial cells suggesting that culturing conditions are critical to maintain a restricted expression pattern for Sca-1. Interestingly, even primary MECs of PyMT tumors cultured on plastic are highly positive for Sca-1 while by *in vivo* imaging and FACS of freshly prepared MECs the tumor epithelium is negative for Sca-1. Thus, an increase in Sca-1 expression when culturing on plastic may be an artifact of the culturing conditions. We now believe, that during culturing, a subset of mammary epithelial cells undergoes epithelial-to-mesenchymal transition (EMT) resulting in the loss of CD24 expression while Sca-1 expression is maintained or gained. The data from Task1 suggest the cells that expressed low levels of Sca-1 and that were positive for CD24 were also enriched for K6 expression and correlated with better outgrowth of normal mammary epithelium than cells expressing high levels of Sca-1. The Sca-1 alone cells (CD24 negative) gave very poor outgrowth and were mostly negative for K6, K14 and K8. Thus, it appears when cultured on plastic, low levels of Sca-1 correlate with greater stem cell activity.

During the 3 years we have analyzed by FACS a number of cell populations in Wnt-1 hyperplasias and tumors and in wildtype mammary glands. The cell surface markers we are using include CD49f, CD24, CD29, Thy1.1, and Sca-1. Using these markers we have been able to further resolve distinct populations that overlap with Sca-1^{low} / $CD24^{High}$ that we previously observed contained either stem cell activity or tumorigenic potential.

Task 2. Generate Sca-rtTA-IRES-tTS and CAG-rtTA-IRES-tTS mice. **Complete**
-No new progress has been made toward this aim in the past year.

Summary of Project to Date:

Over the past 3 years we generated and characterized several transgenic mouse lines with the Chicken-beta-actin (CAG) promoter driving rtTA-IRES-tTS. We initially characterized the CAG-rtTA-IRES-tTS founder lines by isolating tail fibroblasts and infecting them with a retrovirus containing the TRE promoter driving EGFP or mRFP. We observed that infected fibroblasts from several of these transgenic lines did express, in an inducible manner, the TRE reporter. We then bred some of the CAG-rtTA-IRES-tTS expressing mouse lines with the TRE-H2BEGFP lines and treated mice with Dox pellets for several weeks. When we analyzed the mice we did not observe EGFP expression in the Dox treated bigenic lines. We also isolated tail fibroblasts from the bigenic lines and could not detect induction of EGFP in the cultured fibroblasts grown in the presence of dox. Upon discussing these results with another researcher here at UCSF whom attempted to also develop rtTA / tTS bigenic mice (using two separate constructs co-injected into oocytes) I was told that they were not successful at establishing their mouse lines either. Our initial experiments using cell culture studies suggested that rtTA and tTS are compatible and the addition of tTS is required to reduce dox-independent expression of TRE-H2B-EGFP. However, the cells transfected in these experiments

probably had multiple integrations of TRE-H2BEGFP and CAG-rtTA-IRES-tTS resulting in less sensitivity to the presence of tTS because of high expression levels of rtTA. Our transgenic mice most likely had few copies of the transgene and reduced expression of rtTA resulting in tTS repressing rtTA's transactivation capacity.

Since the rtTA-IRES-tTS cassette was used in both the CAG-rtTA-IRES-tTS and Sca1- rtTA-IRES-tTS constructs and it is likely that a similar problem will arise from tTS interference with rtTA we have decided not to pursue the generation of the Sca1-rtTA-IRES-tTS knockin mice using our previously selected ES cell lines. The goal of Task 2 is to develop mouse models that express transgenes in different populations of mammary epithelial cells. The Sca-1 promoter would express transgenes in progenitor cells while the CAG promoter would drive expression in all cells of the mammary epithelium. We feel our best approach is to develop a Sca-1-tTA or Sca-1-rtTA BAC transgenic in collaboration with Dr. Yi Li at Baylor College of Medicine or acquire a Keratin-6-tTA mouse line for expressing transgenes in mammary epithelial progenitors. The alternative strategy for the CAG-rtTA-IRES-tTS transgenic line is described below in Task 5.

Task 3. Generate tetracycline responsive (TRE) H2B-EGFP and H2B-RFP mice. **Complete**

The goal of this Task is to develop a mouse model that can be used to visualize and isolate long surviving/slow turnover cells in the mammary gland. This method is based on data that stem cells have slow turnover in several organs including the mammary gland. Cells that slowly turnover can be identified by using stabilized fluorescent proteins that can be transiently transcribed (pulse) followed by a period when no new protein is generated (chase). During the chase period cells that are actively proliferating or cells that undergo apoptosis will not be labeled leaving a quiescent cell population that retains fluorescence. These cells can be isolated by FACS or visualized in the mammary gland using imaging techniques. To perform these experiments we have obtained the stabilized fluorescent proteins H2B-EGFP and H2B-mRFP, cloned them under the control of the Tet-responsive promoter (TRE) and have generated transgenic mice. H2B-EGFP and H2B-mRFP are histone-2B proteins fused with green and red fluorescent proteins that are highly stabilized.

Several transgenic lines have been generated and were initially characterized by isolating tail fibroblasts and infecting them with a retrovirus that expressed rtTA. Two TRE-H2B-EGFP lines and one TRE-H2B-mRFP line showed inducible expression in the tail fibroblast assay. Further characterization of the two H2B-EGFP lines by breeding with MMTV-rtTA mice revealed that in bigenic mice only one line (w54) expressed in the mammary epithelium in an inducible manner (Figure 1). We are currently expanding this line and breeding mice with MMTV-rtTA to establish a cohort for the experiments in Task 4. We are also breeding the one TRE-H2B-mRFP line with MMTV-rtTA mice to characterize reporter expression.

Task 4. Isolate and characterize H2B-EGFP and H2B-mRFP long-term label retaining cells (LRCs). **In Progress**

The TRE-H2B-EGFP mice described in Task 3 have been bred with MMTV-rtTA mice to generate bigenic heterozygotes. These mice are currently being used to

characterize and isolate mammary epithelial cells that exhibit long-term H2B-EGFP reporter retaining cells.

*Task 5.*Optimize the retroviral/lentiviral infection and transplantation of primary MECs. **Complete**

We tested the efficacy of viral infection and transplantation of MECs to produce outgrowths that express a non-oncogenic gene by infecting primary MECs in monolayer with three different EGFP expressing viral constructs based on either mouse stem cell virus (MSCV), murine moloney leukemia virus (MMLV) or human immunodeficiency virus (HIV). Transduced primary MECs were transplanted into mammary gland-free stroma of 3-week old syngeneic mice and outgrowths were scored for EGFP fluorescence after 8-weeks (Table 1). Transplants of MSCV-EGFP infected MECs exhibited fluorescence in only 21% (6/28) of the outgrowths. Furthermore, the EGFP positive ducts were poorly represented in the 6 transplants that exhibited fluorescence (Figure 2, A and B). Only 5% (± 4%) of the total ductal area in these outgrowths exhibited EGFP fluorescence. Switching viral pseudotype from VSVg to ecotropic did not improve efficiency (data not shown). Similar results were observed with MLV-EGFP (Table 1). HIV-EGFP was slightly more effective and generated 30% (9/30) fluorescent outgrowths with EGFP positive ducts comprising 10% (± 5%) of the total ductal area. In contrast, a virus that co-expressed EGFP with the oncogene *c-myc* (MSCV-Myc) resulted in 62% (10/16) fluorescent outgrowths with nearly the entire ductal area (96% ± 3%) positive for EGFP fluorescence (Table 1 and Figure 2C and D). These data demonstrate that viruses expressing non-oncogenic genes like EGFP are ineffective at producing transgenic outgrowths with the current viral infection and transplantation method.

While preparing infected monolayer cultures for transplantation we observed that MEC colonies gave rise to two morphologically distinct cell populations. Cells located at the periphery of a colony have an elongated appearance and are preferentially infected, while cells in the center of a colony are cuboidal and poorly transduced (Figure 2E, arrow). We separated the peripheral and central cells by differential trypsinization and performed limited dilution transplantations to identify the population with highest MaSC activity. Central cells exhibited four times greater outgrowth capacity than peripheral cells (Figure 2F). Thus, the MEC population with the greatest stem cell activity is poorly targeted by the monolayer infection method.

We next reasoned that infection of primary MECs in suspension might enhance the transduction of MaSC. In addition to increasing the cell surface area this method allows for a reduced culturing volume that effectively raises the viral titer. Since the suspension infection method does not require prior culturing we tested both freshly prepared MECs in addition to central cells. Primary MECs were infected overnight in suspension with HIV-EGFP and formed large multi-cellular aggregates consisting of both myoepithelial and luminal epithelial lineages (Figure 2G). Cells that failed to aggregate were enriched for stromal, apoptotic, and blood cell markers. Outgrowths of HIV-EGFP infected aggregates exhibited fluorescence in 62% (15/24) of the transplants with EGFP positive ducts comprising 72% (±28%) of the total ductal area (Figure 2 H-I and Table 1), a

considerable improvement over the monolayer infection method. Aggregates derived from HIV-EGFP infected central cells gave rise to outgrowths with comparable results (Table 1). In contrast, MLV-EGFP infected aggregates from freshly prepared MECs produced fluorescent outgrowths in only 4% (1/27) of the transplants with 7% of the ductal area positive for fluorescence. Only a small increase in the number of EGFP positive outgrowths (4/26) was observed when central cells were transduced. Our data show that infecting MECs in suspension with a HIV-based virus increases the representation of transduced cells in outgrowths and appears to target MaSCs more efficiently than the monolayer infection method.

MaSC are both highly regenerative and multipotent; therefore, if MaSCs are targeted by the modified infection method then transduced cells should contribute to both myoepithelial and luminal epithelial cells in transplants and give rise to secondary outgrowths. We characterized the expression of EGFP in primary outgrowths to establish whether transduced MECs contribute to both cellular compartments of the mammary bilayer and form lobulo-alveoli during pregancy. EGFP was detected in myoepithelial cells and in both the estrogen receptor positive and negative cells of the luminal epithelium (Figure 3 A-C). Transduced MECs also developed into lobulo-alveoli in pregnant recipients (Figure 2D). We next assessed the regenerative capacity of infected MECs using a secondary transplantation assay. Small ductal fragments from lentiviral-infected primary outgrowths were transplanted into secondary hosts and broad EGFP fluorescence was observed throughout the ductal network of virgin recipients and in lobulo-alveoli of pregnant hosts (Figure 3 E and F). Using FACS we analyzed whether transduced MECs can contribute an enriched mammary stem cell population in primary and secondary outgrowths. Stingl and colleagues recently isolated three distinct MEC populations by FACS using antibodies against the cell surface proteins CD24 (heat stable antigen) and CD49f (alpha-6 integrin). These populations are the MaSC containing mammary repopulating units (MRU), myoepithelial cells (myo) and mammary colony forming cells (ma-CFC). FACS analysis of primary and secondary outgrowths revealed that infected cells are present in all three populations (Figure 3G " Primary" and "Secondary"). Collectively, these data suggest that lentiviral transduced MECs are regenerative and can comprise all major cell types of the mammary gland including the stem cell enriched MRU population.

In limited dilution transplants a single MaSC can give rise to a complete mammary outgrowth. We examined whether outgrowths from the suspension infection technique are derived from more than one progenitor cell. Freshly prepared MECs were separately infected with either a lentivirus that expressed a red fluorescent protein (pEIR) or green fluorescent protein (pEIZ). Infected MECs were mixed at a ratio of 1:1 and a total of 200,000 cells were injected into cleared fat pads. In outgrowths some ducts exhibited uniform fluorescence of a single marker and were either red (Figure 3 H-I, large arrowhead) or green (Figure 3 H-I, arrow). Other ducts had a segmented pattern and displayed regions of uniform fluorescence (Figure 3 H-I, small arrow) and regions of intermixed green and red fluorescence (Figure 3 H-I, large arrow). These data demonstrate that when a non-limiting dilution of cells is used then ducts in outgrowths can develop from more than a single infected progenitor.

Task 6. Use the optimized technique for infection of MECs to study the role of Kuzbanian in mammary gland development. **In Progress**

Notch is composed of a family of four transmembrane receptors that interact with the ligands Delta, Serrate and Jagged. Upon ligand interaction, Notch undergoes a multi-step cleavage process that is initiated by ADAM-10 (Kuzbanian) to release the intracellular domain of the Notch receptor (Nicd). Nicd then travels to the nucleus where it interacts with CBF-1 in a complex that converts CBF-1 from a transcriptional repressor to an activator. The Nicd/CBF-1 complex activates a number of genes that are involved in cell fate decisions.

Notch signaling is involved in cell-fate determination of the mammary gland and overexpression of the intracellular domain of Notch4 can function as an oncogene. To block Notch signaling we chose to express a dominant-negative form of kuzbanian in the mammary gland. However, due to problems with cloning a wild-type kuzbanian, where mutations were observed after propogation in bacteria, we chose a different route to investigate. Suppressor of hairless (XSu(H)) is the *xenopus* homologue of CBF-1 and a dominant negative form of XSu(H) (dnXSu(H)) that contains a mutation in the DNA-binding domain has been described to block mammalian Notch signaling. We chose to block Notch signaling through the expression of dnXSu(H) in MECs. Primary MECs were infected by suspension infection with pEIZ-dnXSu(H). Outgrowths were analyzed 5-weeks after transplantation when terminal end buds (TEBs) are still present. TEBs are specialized structures that form at the distal tips of developing ducts that are important for ductal morphogenesis. Outgrowths from dnXSu(H) infected MECs exhibited severally dysplastic terminal end buds and distended primary ducts (Figure 4 A-A'''). Normal histological morphology was observed in TEBs from control pEIZ infected MECs while TEBs in pEIZ-dnXSu(H) outgrowths exhibited filled lumens (Figure 4 B and C). Furthermore, 5-weeks after transplantation the outgrowths from pEIZ-dnXSu(H) infected MECs occupied less than half of the area occupied by pEIZ alone (Figure 4 D). These data suggest that Notch signaling is important for normal mammary morphogenesis. Further experiments are ongoing to investigate the role of Notch signaling on the cell-fate determination of MECs and in the functional differentiation of the mammary gland during pregnancy.

TRAINING

During the past year I attended the Gordon Research Conference on Mammary Gland Biology where made a platform presentation on targeting mammary stem cells using lentiviral vectors. I also attended an informal seminar series at UCSF to discuss our research on mouse models of mammary cancer. These meetings are held twice a month. I also participated in laboratory meetings, attended local seminars and trained rotating students.

KEY RESEARCH ACCOMPLISHMENTS:

- Development and characterization of TRE-H2B-EGFP transgenic mice.

- Development of a technique to target mammary stem cells using lentiviruses for the rapid analysis of gene function in the mammary gland.

REPORTABLE OUTCOMES

Bryan Welm, Gerrit Dijkgraaf and Zena Werb. Targeting Mammary Gland Stem Cells with Viruses for Analysis of Gene Function during Mammary Development. Gordon Research Conference, Mammary Gland Biology (2006)

CONCLUSION

Understanding the etiological events that can give rise to tumors is critical for prevention, early diagnoses and treatment of breast cancer. The studies being performed here will elucidate cell populations that are targeted by specific oncogenic pathways. We have observed that tumors can be divided into progenitor and luminal phenotypes suggesting that oncogenic events can target specific differentiation pathways. These data suggest that diverse mechanisms and cell populations can contribute to tumorigenesis. The results of this work may aid in the development of new therapeutics that target specific cell populations or differentiation pathways. Our research to date has identified CD24 was as a marker expressed on a subset of Sca-1 MECs that can be used to separate cell populations with enhanced outgrowth potential. The significance of identifying markers coexpressed on Sca-1 cells allows us to further enrich the Sca-1 cell population for stem cell activity. By sorting with Sca-1 and CD24 markers we have characterized several MEC populations for differentiation factors and outgrowth potential. Once a highly enriched stem cell population is identified then future studies can address how these cells contribute to mammary tumorigenesis.

We have also developed a number of useful viral constructs and developed an efficient primary MEC transduction and transplantation technique that can be used to study the effects of genes on mammary development and tumorigenesis. These methods will be useful not only for our studies but should also benefit many investigators in the mammary gland biology research community.

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FIGURE LEGENDS

Table 1: The efficiency of MSCV, MLV and HIV based viruses to produce transgenic mammary outgrowths from the centrifugation-assisted or suspension infection methods.
*Outgrowths were scored as positive for EGFP only if a discernible duct could be observed by epifluorescence.
^Standard deviation is shown in parentheses.

Figure 1: Frozen sections of mammary glands from MMTV-rtTA/TRE-H2B-EGFP mice treated with or without doxycline (dox) in their drinking water for 1 week. H2B-EGFP was observed in the mammary epithelium of dox treated mice.

Figure 2: (A) Representative carmine-alum stained outgrowth from primary MECs transduced by centrifugation-assisted infection using the MSCV-EGFP virus. LN indicates lymph node and the asterisk indicates the site of injection. Scale bar is 1mm. Major ducts are traced in black to visualize the extent of outgrowth. The boxed area represents the location of the highlighted epifluorescence in B. (B) Epifluorescence in MSCV-EGFP outgrowth. Scale bar is 1mm. (C) Representative outgrowth from primary MECs infected with MSCV-Myc. (D) Epifluorescence of the boxed region in panel A. (E) A monolayer of a primary MEC colony derived from a single ductal fragment that was grown for 96 hours. The primary cells were infected using the centrifugation-assisted method with MLV-EGFP at 24 and 48 hours after culturing. The asterisk shows the position where the ductal fragment originally attached and subsequently spread. Propidium iodide stained nuclei are red and infected cells are green. The arrow indicates the peripheral cells that are preferentially infected. A demarcation of highly infected and poorly infected cells is observed between the central cells (surrounding the asterisk) and the peripheral cells (arrow). Scale bar represents 0.25mm. (F) Limited dilution transplantation of enriched peripheral or central cells. Peripheral and central cells were enriched by differential trypsinization and between 2000-5000 cells were transplanted into the cleared fat pads of mice. The percent of the transplants that contained outgrowths was quantified. (G) Central cells were isolated as single cells by differential trypsinization and were infected with HIV-EGFP in suspension for 16 hours. Cells were imaged after 72 hours of aggregation. Green shows the infected cells within the aggregates. (H) Representative outgrowth from transplanted aggregates that were infected with HIV-EGFP in suspension culture. (I) Epifluorescence of ducts within the boxed area. Scale bar represents 1mm.

Figure 3: (A and B) Sections of primary outgrowth tissue derived from HIV-EGFP infected MECs were immunostained for EGFP (green) and cytokeratin-14 (B, red). EGFP

is expressed in both myoepithelial (arrowheads) and luminal (arrow) epithelial cells. (C) Cross-section of a duct from HIV-EGFP infected outgrowth showing EGFP (green) in both estrogen receptor positive and negative cells (red) of the mammary epithelium. Scale bar represents 50um. (D) Epifluorescence from a primary outgrowth of HIV-Zsgreen infected MECs in a pregnant recipient showing fluorescence in the lobulo-alveoli. (E) Secondary outgrowth from HIV-EGFP infected MECs showing EGFP fluorescence in ducts and TEBs (arrow). (F) Secondary outgrowth from a pregnant mouse exhibiting EGFP fluorescence in lobulo-alveoli. (G) FACS analysis of the CD24 and CD49f populations in uninfected MECs (Uninfected MECs) and EGFP gated cells isolated from primary (Primary) and secondary (Secondary) outgrowths. Gates are indicated for the mammary colony forming cells (MaCFC), mammary repopulating units (MRU) and myoepithelium (Myo). (H-J) Outgrowth from a representative mixed cell transplant showing H2B-mRFP fluorescence alone (H), Zsgreen fluorescence alone (I) and the merge (J). The large arrowhead indicates a duct that is positive for H2B-mRFP alone and the small arrowhead indicates a duct that is positive for Zsgreen alone. The large arrow indicates a segment of a duct that has interspersed Zsgreen and H2B-mRFP fluorescence and the small arrow shows a segment of the same duct that is only Zsgreen positive. Scale bars A, B, H-J are 50um, C and D are 100um, and E-G and L-N are 1mm.

Figure 4: (A) 5 week outgrowth from pEIZ-dnXSu(H) infected MECs. Higher magnification of the boxed areas are shown in A'-A'''. The scale bar represents 1mm. (A' and A'') Examples of large dysplastic terminal end buds. (A''') This panel shows an example of a distended primary duct. (B) Cross-section of a TEB in an outgrowth from control pEIZ infected MECs showing a lumen (lu) surrounded by the multi-layered body cells. (C) Cross-section of a TEB from in a pEIZ-dnXSu(H) infected outgrowth showing an expanded body cell region and small lumen. (D) The relative fat pad area occupied by control pEIZ and dnXSu(H) outgrowths showed that transplants of dnXSu(H) infected MECs occupied less than half the area of pEIZ outgrowths.

Table 1

Infection Method	Virus Type	Gene Expressed	Cell Preparation	EGFP Positive Outgrowths*	Percent of Outgrowth Area Positive for EGFP^
Centrifugation	MSCV	EGFP	Cultured	21% (6/28)	5% (±4%)
	MLV	EGFP	Cultured	13% (4/30)	3% (±2%)
	Lentivirus	EGFP	Cultured	30% (9/30)	10% (±5%)
	MSCV	Myc and EGFP	Cultured	62% (10/16)	96% (±3%)
Suspension	MLV	EGFP	Fresh	4% (1/27)	7%
	MLV	EGFP	Central	15% (4/26)	13% (±15%)
	Lentivirus	EGFP	Fresh	62% (15/24)	72% (±28%)
	Lentivirus	EGFP	Central	93% (26/28)	57% (±30%)

Figure 1

MMTV-rtTA/TRE-H2B-EGFP Bigenics

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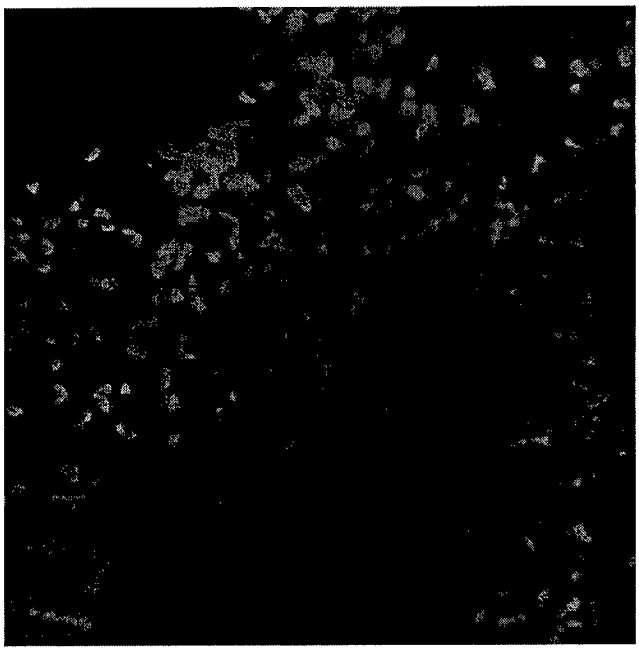

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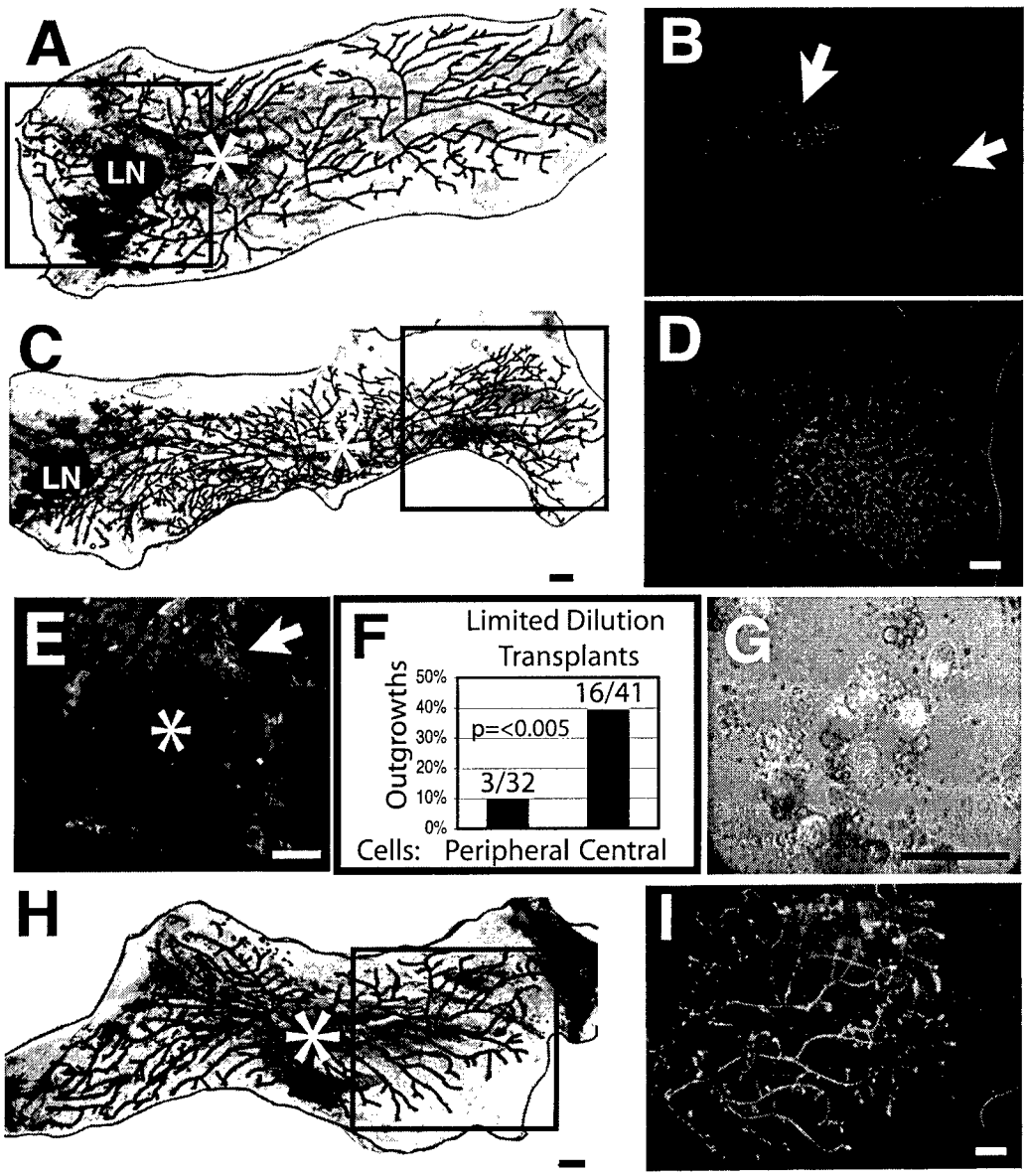
Figure 2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Limited Dilution
Transplants
Outgrowths
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Figure 3

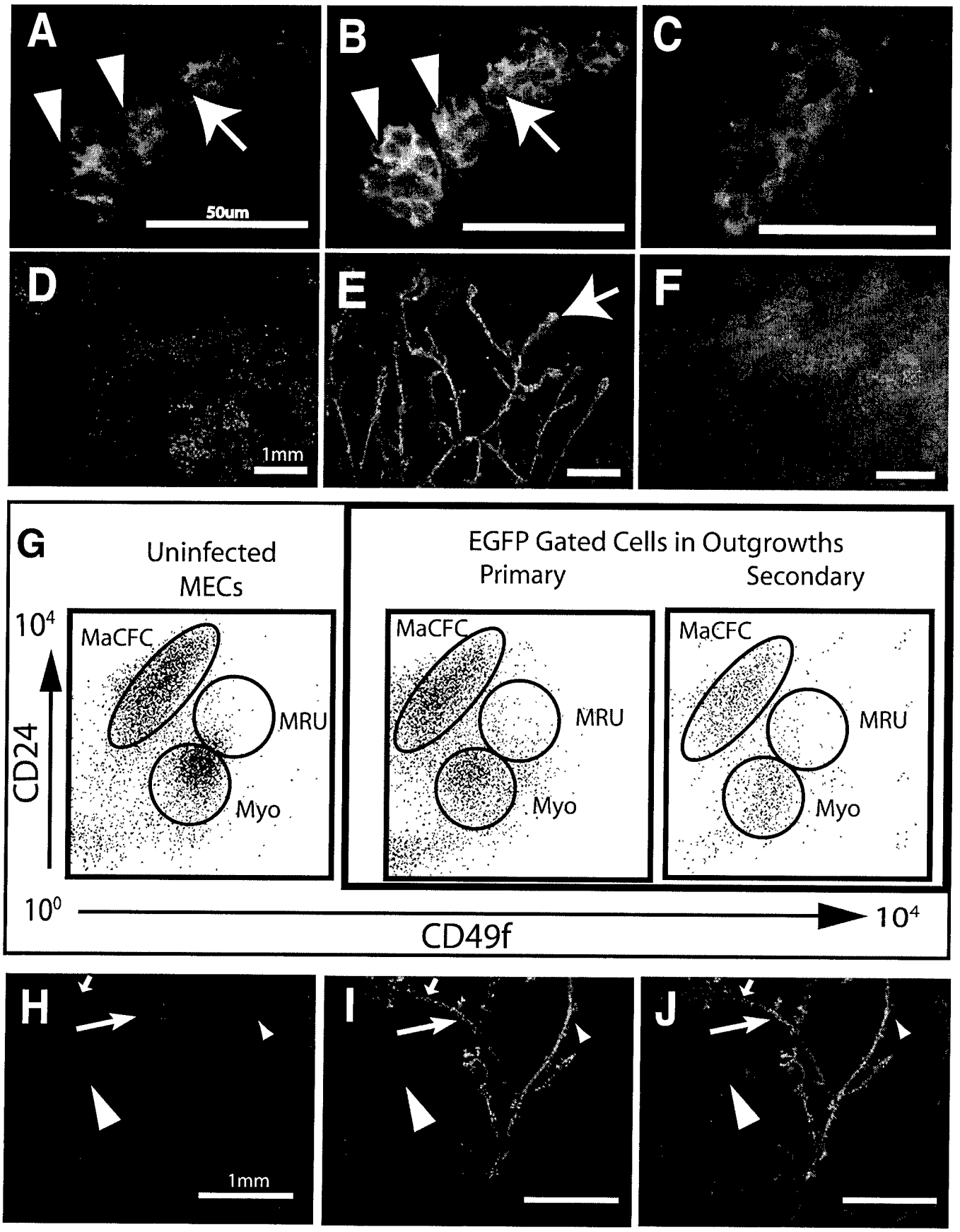
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Figure 4