AN APPROACH TO IDENTIFY TARGETS OF BREAST CANCER STEM CELLS

A FUNCTIONAL GENOMICS APPROACH TO IDENTIFY NOVEL THERAPEUTIC TARGETS OF MAMMARY TUMOUR-INITIATING CELLS

By

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To my family,

this work has been for you.

ABSTRACT

Much interest has recently accumulated of the role of adult stem cells in both normal tissue homeostasis and carcinogenesis. Whereas normal and cancerous mammary epithelial stem cells have been identified and isolated from bulk primary tissue, little remains known about their regulation *in vivo*. Here we describe the molecular profile of mammary epithelial stem cells cultured *in vitro* and that of their tumourigenic counterparts, breast cancer stem cells. Our studies of gene transcription reveal potential mechanisms that may cooperate in the regulation of normal and cancer stem cells *in vitro*, and may also reflect their *in vivo* behaviour. These data bear consequences for the design of novel breast cancer therapeutics, as cancer stem cells are thought to resist conventional treatments and persist thereafter, causing disease relapse and seeding metastases. To address this issue we have devised a functional genomics approach to screen for novel biomarkers and therapeutic targets of breast cancer modeled *in vitro*; this culture system is centered on *bona fide* stem cells and may therefore offer improved relevance to human disease when compared with breast cancer cell lines.

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MULTIPLE AUTHOR CONTRIBUTIONS

The data reported in this thesis includes contributions from others. Microarray expression profiling, including cell culture and RNA isolation, was performed by Maria Barcelon (mammospheres), Shahab Shahnazari (tumourspheres), and Gina Fidalgo (primary mammary and tumour tissue). RNA was hybridized to Affymetrix GeneChips at the Ottawa Genome Centre. Data files were imported, manipulated, and analyzed by David Gludish. Contributions from these individuals therefore includes: Figures 1, 2, 4, 8, 9, and 10, and Tables 1, 2, and 3.

Other contributions:

Figure 3AB - David Gludish and Maria Barcelon performed immunocytochemistry in parallel

Figure 6AB - Natasza Kurpios

Figure 11 - Primary tissue was isolated and fixed by Natasza Kurpios and Adele Girgis-Gabardo. Immunohistochemistry was performed by David Gludish.

PREFACE

The following dissertation is divided into two chapters and describes a functional genomics approach with the global objective to identify and target breast cancer stem cells. An introduction is given for each chapter to provide background for mammary gland biology, a comprehensive summary of the current literature in the area of normal and cancer stem cell research, and an overview of functional genomics. The first chapter details the global transcriptional profile of mammary epithelial cells and of mammary tumour cells, revealing a "stemness" gene signature expressed in both cell populations, in support of the cancer stem cell hypothesis. Based on the information generated in Chapter I, the second chapter describes a data-mining strategy to define biomarkers of breast cancer, applying a functional genomics approach to test their potential as therapeutic targets. The feasibility of RNAi technology to silence oncogenes is demonstrated.

CHAPTER 1

TRANSCRIPTIONAL PROFILING OF MOUSE MAMMARY EPITHELIAL CELLS: A STEMNESS GENE SIGNATURE FOR MAMMOSPHERES AND TUMOURSPHERES

INTRODUCTION

Mammary gland biology

The murine mammary gland is embedded in a pad of fat and stromal cells, and at birth consists of a primitive system of branched epithelial ducts containing a continuous hollow lumen (Hogg *et al.*, 1983) that ends and opens at the nipple. The mammary gland is a unique developmental system as much of its development occurs post-natally, beginning during puberty at about 5 weeks of age. Fueled by cell proliferation (Bresciani, 1965), the mammary ducts of a pubertal mouse expand and invade the fat pad with bulbous terminal end buds (Silberstein and Daniel, 1982), undergo morphogenesis, and by sexual maturity at 8-10 weeks have formed a network of subtending ducts defined by a bilayered epithelial sheet (Stingl *et al.*, 2005). The hollow interior (lumen) of each mammary duct is lined by an inner layer of luminal epithelial cells surrounded by an outer layer of myoepithelial cells (Silberstein, 2001).

Following puberty, the mammary gland enters a resting state that persists until pregnancy. Hormone signals of pregnancy stimulate the formation of alveolar buds that develop at regular intervals along the surface of the subtending ducts; these buds extend (Brisken and Rajaram, 2006), invading and filling the mammary fat pad in preparation for milk production. Luminal cells connect tightly to maintain a leak-free duct (Daniel *et al.*, 1995). Lactogenic hormones signal the differentiation of specialized luminal cells called alveolar cells that are responsible for secreting milk proteins into the lumen (Brisken and Rajaram, 2006). Myoepithelial cells attach the luminal cells to the basement membrane,

and are connected laterally to each other (Daniel *et al.*, 1995); during lactation, myoepithelial cells contract to collapse the lumen and expel milk from the alveoli through the ducts (Adriance *et al.*, 2005).

Following the cessation of suckling by weaning, the mother's ducts swell with milk (Wilde *et al.*, 1999), signaling involution - the death by apoptosis of most epithelial cells in the mammary gland and concurrent remodeling of the fat pad (Wilde *et al.*, 1999). Following involution, the mammary gland is returned to a state similar to that of the virgin gland until the next pregnancy (Daniel and Smith, 1999); this cycle of tissue regeneration can be repeated many times throughout the life of a female mouse.

Functional evidence for mammary epithelial stem cells

Similar to other regenerating tissues like skin and the intestinal lining (Cotsarelis, 2006), the proliferative potential of the mammary gland is governed by a population of adult stem cells that are present following development and persist throughout the life of the animal (Smith and Chepko, 2001). The existence of these mammary epithelial stem cells was first shown in elegant transplant experiments performed decades ago (Deome *et al.*, 1959) wherein the mammary fat pads of virgin female mice were surgically cleared of existing epithelium and then injected with primary mammary epithelial cells from donor syngeneic mice (orthotopic allograft). To stimulate proliferation of the graft, recipient females were put through pregnancy and their fat pads were harvested at parturition, revealing a reconstituted mammary ductal and branching structure; this process could be

repeated multiple times demonstrating a cell population with extensive self-renewal capacity (Daniel, 1973). However, despite the power and relative simplicity of this experimental design, it has proven difficult to identify, isolate and characterize mammary epithelial stem cells (Dontu *et al.*, 2003b).

By definition, stem cells live somewhat indefinitely within adult mammalian tissue, divide rarely (Furukawa, 1998), and possess the autonomy of self-renewal, that is, cell division without commitment to differentiation (Liu *et al.*, 2005). Under normal conditions, stem cell self-renewal is regulated by the interaction of these cells with their niche (Fuchs *et al.*, 2004), a defined environment that signals to the stem cell about the status of the surrounding tissue (Dor and Melton, 2004). Such signals are difficult to study *in vivo*, and thus methods to isolate and study stem cells *in vitro* may allow the characterization of discrete signaling pathways that govern their maintenance, self-renewal, and differentiation (Dontu *et al.*, 2003a; Stingl *et al.*, 2006a). However, stem cells are prone to differentiate when removed from their niche (Scadden, 2006), complicating their analysis *in vitro* (Simon-Assmann *et al.*, 2007).

A system to culture and study stem cells *in vitro*

It was first discovered that when cultured *in vitro* with EGF (epidermal growth factor) and bFGF (basic-fibroblast growth factor), primitive neural cells give rise to non-adherent "neurosphere" suspension colonies (Reynolds and Weiss, 1992; Vescovi *et al.*, 1993). Neurospheres are cultured in serum-free stem cell media and consist of

undifferentiated neural stem cells (Reynolds and Weiss, 1996) that can be induced to form the various differentiated cell types of the mature nervous system. Following these landmark experiments, we employed a similar approach to propagate normal mouse mammary epithelial stem cells *in vitro* (Kurpios, 2005). Under these conditions, primary mammary cells give rise to non-adherent "mammospheres" that are clonal in origin and which are composed of progenitors and stem cells. These points were demonstrated by the engraftment of mammospheres into the cleared fat pad of recipient mice, followed by stimulation with pregnancy. Echoing decades-old, classical studies of mammary gland stem cells these transplants result in outgrowths resembling normal mammary gland structure (Daniel, 1973; Deome *et al.*, 1959; Kurpios, 2005; Personal communication: Kurpios and Hassell, 2006). Furthermore, mammosphere-derived outgrowths secrete milk, demonstrating the utility of mammospheres to culture primitive mammary epithelial cells that are capable of multi-lineage differentiation *in vivo*. Importantly, tissue fragments removed surgically from mammosphere-derived outgrowths can be serially passaged *in vivo;* these grafts give rise to a full mammary structure through each successive orthotopic transplant, demonstrating the presence within mammospheres of *bona fide* mammary epithelial stem cells capable of extensive self-renewal (Kurpios, 2005). Importantly, mammosphere cells can also be induced to differentiate *in vitro* by culturing them in complete medium that contains serum and lacks growth factors EGF and bFGF. This process may reflect their behaviour during differentiation *in vivo*, and thus mammospheres may serve as a valuable tool to study normal mammary gland function *in vitro*.

Adult stem cells may be targets for oncogenic transformation

Irrespective of their function, every adult tissue stem cell is maintained in an undifferentiated state by cells and signals in its immediate vicinity, presumably over a period of years or longer. Thus, it seems that adult stem cells are ideal candidates to accumulate genetic lesions over time, eventually resulting in oncogenic transformation (Tan *et al.*, 2006). Since stem cells are armed with extensive proliferative capabilities and may be intrinsically resistant to apoptosis (Mueller *et al.*, 2006; Young *et al.*, 1999), perhaps these cells need only collect mutations that relieve them of regulation by their niche for transformation (Moore and Lemischka, 2006). If true, an adult stem cell bearing lesions within developmental or mitogen-responsive signaling pathways appears the ultimate perpetrator of oncogenesis.

If cancer arises in stem cells, a hypothesis that is compatible with the "multiple hit" model of carcinogenesis, it is conceivable that the resulting tumour would display a heterogeneous cell population reflective of normal tissue differentiation. Indeed, there exists a cellular hierarchy within many human malignancies that often mimics the cytoarchitecture of the adult tissue in which the tumour arose (Wang and Dick, 2005). These findings supported a role for stem cells in the classical view of transformation and spawned the earliest incarnation of the cancer stem cell hypothesis, which in part sought to account for the heterogeneity observed among cells resident in a presumably clonal tumour (Bruce and Van Der Gaag, 1963). Thus, it seemed that cancer stem cells could be fundamentally defined as those cells at the top of the hierarchy, with the capacity to

differentiate, albeit aberrantly, into phenotypically distinct progeny that populate the tumour. However, the cancer stem cell hypothesis has evolved more recently to accommodate increasing evidence that this cellular heterogeneity may delineate functional subpopulations of cancer cells (Tan *et al.*, 2006).

The revised Cancer Stem Cell hypothesis

Normal adult stem cells are rare, divide infrequently, and are the only cells able to regenerate their tissue of residence, functioning in tissue repair and homeostasis (Dor and Melton, 2004). Similarly, in addition to being able to recapitulate phenotypically diverse progeny, it is possible that cancer stem cells are the only cells in a given tumour with the ability to initiate (or re-initiate) the disease (Wang and Dick, 2005). Thus, perhaps cancer stem cells are better defined functionally, as tumour-initiating cells (Neuzil *et al.*, 2007). Consistent with this notion, it was found that only a rare subset of human acute myeloid leukemia cells could transfer the disease into naïve immunodeficient mice in xenograft assays (Bonnet and Dick, 1997; Lapidot *et al.*, 1994). Thereafter multiple groups found tumour-initiating cells at low frequency in a variety of human solid tumours (Al-Hajj *et al.*, 2003; Collins *et al.*, 2005; Kim *et al.*, 2005; Li *et al.*, 2007a; Prince *et al.*, 2007; Singh *et al.*, 2003; Singh *et al.*, 2004b). Secondary tumour xenografts often mimic the hierarchy observed within the primary tumour of origin, raising the possibility that rare tumour-initiating cells are cancer stem cells that maintain the potential to differentiate into multiple classes of progeny. These results suggest that

like normal stem cells, cancer stem cells may also divide infrequently, fueling tumour growth through the expansion of their descendants, transformed progenitors or transit amplifying cells. It has been suggested that the aberrant differentiation of these transformed progenitor cells results in the cellular heterogeneity observed in human tumours (Bonnet and Dick, 1997; Singh *et al.*, 2004a).

The identification of cancer stem cells has therapeutic implications. Global observations about normal stem cells may help to explain why conventional therapies targeting the bulk tumour cells often fail: chemo- and radiotherapy may spare rare cancer stem cells, leading to disease relapse (Wicha *et al.*, 2006). Resistance to radiation has been reported for normal and cancer stem/progenitor cells in brain and bone marrow (Hambardzumyan *et al.*, 2006; Mahmud *et al.*, 2005), and in mammary epithelial progenitor cells, measurable radio-resistance seems to be conferred by β-catenin activation (Woodward *et al.*, 2007). Furthermore, cancer stem cells may be responsible for seeding metastases at sites distant from the original tumour making their characterization a prerequisite for the design and validation of effective therapeutics (Hendrix *et al.*, 2007; Li *et al.*, 2007b). However, by their nature cancer stem cells are rare in human tumours (as measured by widely employed xenograft transplant assays in immunodeficient NOD-SCID mice), complicating the study of these cells.

Mouse tumour-initiating cells propagated *in vitro*

Increasing evidence suggested the existence of cancer stem cells in human tumours, though their observed frequency was low. Since primary tumours from transgenic mice might provide a more abundant and renewable source of cancer stem cells, our lab previously attempted to culture these cells *in vitro* under conditions similarly employed for neurospheres and mammospheres (Kurpios, 2005; Reynolds and Weiss, 1992). In these previous studies, we found that mouse MMTV-Neu tumours (Guy *et al.*, 1992), resulting in part from the overexpression of the Her2/Neu receptor tyrosine kinase, gave rise to tumourspheres *in vitro*; the cells comprising tumourspheres could re-initiate the disease after orthotopic transplant in syngeneic hosts (Kurpios, 2005). Surprisingly, we found that very few tumoursphere cells were required to seed new tumours, in contrast to results previously obtained with cells from human tumours (Al-Hajj *et al.*, 2003). Subsequent data from our lab showed that both tumourspheres and the primary tumours they derive from bear a high fraction of tumour-initiating cells, and are therefore valuable to study parameters of the cancer stem cell hypothesis (Kurpios, 2005).

Here we report that mammospheres and their differentiated counterparts yield rich studies of global gene expression, detailing changes in mRNA abundance induced during their *in vitro* differentiation programme. Markers of adult stem cells including CD49f (α6-integrin) and CD34 (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002) are expressed at high levels in mammosphere-resident cells, while their expression is all but

extinguished in cells induced to differentiate. Likewise, well-known markers of mammary epithelial differentiation such as α-smooth muscle actin are expressed at low basal levels in mammospheres and their expression is significantly upregulated during their *in vitro* differentiation programme, helping to validate this *in vitro* model as a useful surrogate for studying mammary gland differentiation. We have also identified candidate stemness genes involved in mammary epithelial stem/progenitor cell maintenance and self-renewal, suggesting that mammospheres may also be used to study these processes. Moreover we describe the gene expression profiles of mouse tumourspheres in comparison with mammosphere cells and those induced to differentiate *in vitro*. Our results suggest that tumourspheres exhibit an amplified stem cell phenotype by comparison to mammospheres. We propose experiments to use the tumoursphere system as a tool to test many facets of the cancer stem cell hypothesis, including the involvement of candidate genes in tumoursphere self-renewal or tumourigenic potential.

RESULTS

A mammosphere culture system to investigate mammary gland development *in vitro*

We previously showed that cultured mammospheres contain functional mammary epithelial stem cells capable of self-renewal and multi-lineage differentiation, and may therefore serve as a useful surrogate for *in vivo* mammary gland development (Kurpios, 2005). We hypothesized that defining their global transcriptional profile would reveal mechanisms of mammary stem cell renewal or differentiation. To this end our lab

previously derived three replicate mammosphere cultures, where each culture was a pool of mammary glands from three virgin FVB mice aged 5-7 weeks (Personal communication: Kurpios and Hassell, 2006). Total cellular RNA was isolated from mammospheres and their descendant cells induced to differentiate *in vitro* (differentiated mammospheres), and was prepared as required by Affymetrix for hybridization to their MOE430 GeneChip microarrays (see Materials and Methods). Judged by present calls in each of the three replicate samples, mammospheres expressed 10,427 transcripts, roughly half of the 22,690 total transcripts on the MOE430A chip. Similarly, 8,470 genes were expressed in differentiated mammosphere cells.

During the transition to *in vitro* tissue culture, cells isolated from primary animal tissue can undergo gross changes in gene expression and cellular phenotype. Hence to exclude the possibility that genes expressed in mammospheres or differentiated cells were induced as an artifact of cell culture, genes expressed in these samples were compared with the global gene expression profiles of primary mouse mammary glands. In an effort to capture a cohort of transcripts representative of mammary gland development and overall homeostasis, total RNA deriving from mammary glands of virgin mice aged 5 weeks (pubertal), 10 weeks (sexual maturity), and 15 weeks (resting mammary gland) was prepared and hybridized to MOE430A chips as described above. Six chips in total – two biological replicates for each timepoint – were hybridized with pools of RNA isolated from the mammary glands of 5 mice each. The outcome of these Affymetrix GeneChip analyses is reported in Figure 1A. As assessed by consistent present

calls in each cell compartment, 5286 genes were expressed in common among mammospheres, those induced to differentiate, and the primary mammary gland. A total of 2797 transcripts were found to be expressed by cells cultured *in vitro* (mammospheres and differentiated cells) but not *in vivo*. Likewise, 553 transcripts were expressed by mammary tissue *in vivo*, but not by cultured cells *in vitro*. Thus, greater than eighty percent of all 6305 genes expressed in the mammary gland were also expressed in mammospheres and those induced to differentiate, supporting our claim that mammospheres may serve as a model to study normal mammary gland processes *in vitro*.

To better understand the molecular profile of mammospheres, we further analyzed the 10,427 transcripts expressed in these cells. We found that 950 genes were preferentially expressed in mammospheres at levels at least twofold higher than in mammosphere cells induced to differentiate *in vitro* (Table S1); 395 of these 950 transcripts were reproducibly expressed in the primary mammary gland (Figure 1C). Also, among these 950 genes preferential to mammospheres, 440 were not detectably expressed in at least one of three replicate samples from mammospheres induced to differentiate, further confirming that these genes are negatively regulated during *in vitro* differentiation.

The Gene Ontology consortium curates the literature to organize all genes (in the mouse genome, for example) into categories which classify their function. Thus to assist in assigning an overall phenotype to mammosphere-derived cells, we performed ontological clustering in which the 950 transcripts preferential to mammospheres were

used to query gene ontology Biological Process categories. Overall, the mammosphere expression profile was consistent with a proliferating cell population capable of extracellular matrix remodeling. Specifically, processes such as cell cycle, DNA replication, and collagen catabolism were overrepresented in mammospheres (Table 1, left column). Conversely, the expression profile of differentiated mammospheres was consistent with cellular morphogenesis, homing, homeostasis and energy production; significant overlap was found with specific processes such as cell adhesion, chemotaxis, cell death, and generation of precursor metabolites and energy (Table 1, right column).

Mammospheres preferentially express genes characteristic of stem cells

Our lab previously demonstrated the existence of mammary stem cells within mammospheres and proposed that genes preferential to these cells include markers of stem/progenitor cells (Kurpios, 2005). Indeed, mammospheres express the cell surface proteins used to enrich for primary mammary epithelial stem cells, a subpopulation identified recently as $CD24^{-/low}$ and either $CD49f^{high}$ or $\beta1\text{-integrin}^{high}$ (Shackleton *et al.*, 2006; Stingl *et al.*, 2006a). Interestingly, we discovered that mammospheres exhibit high preferential expression of mammary epithelial stem cell marker CD49f/integrin-α6 (32-fold), mammary stem cell fate regulator Notch-1 and its ligand Delta-like-1 (3.4-fold and 6.1-fold, respectively) (Dontu *et al.*, 2004), stemness gene tenascinC (2.3-fold), and hematopoietic stem cell markers CD34 (8.3-fold), CD38 (3.2-fold), and c-Kit (4.6-fold) (Baum *et al.*, 1992; Civin *et al.*, 1996; Hao *et al.*, 1998; Ikuta and Weissman, 1992;

Terstappen *et al.*, 1991; Uchida and Weissman, 1992). Mammospheres also express the breast/prostate cancer stem cell marker CD44 (Al-Hajj *et al.*, 2003; Collins *et al.*, 2005) and the neural/brain tumour stem cell marker CD133/Prominin-1 (Singh *et al.*, 2003; Singh *et al.*, 2004b).

These observations prompted us to further inquire whether mammospheres share more significant overlap with other stem cell populations, with the goal to establish a set of genes in common which may globally determine the stem cell phenotype. Notably, two groups have reported the global gene expression profiles of mouse neural, embryonic, and hematopoietic stem cells (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002), while two other groups characterized the transcriptional profile of murine hair follicle stem cells (Morris *et al.*, 2004; Tumbar *et al.*, 2004). Thus we compared the 950 transcripts preferential to mammospheres with the genes common to these other stem cell populations (Figure 2). "Stemness" genes, a consensus set of transcripts commonly enriched in neural, embryonic, and hematopoietic stem cells, shared significant overlap with genes preferential to mammospheres - 40 transcripts or 4.21% (Figure 2A). Included in these overlapping genes were mammary epithelial stem cell marker CD49f/integrin-α6 (Stingl *et al.*, 2006a), transcription factor E2F5, β-catenin stabilizer Pin1 (Ryo *et al.*, 2001) and the direct β-catenin target gene CyclinD1 (Rowlands *et al.*, 2003), and TGFβ target gene TGFb1i4 (Shibanuma *et al.*, 1992). The strength of this overlap suggests that, similar to other stem cell populations, genes preferential to mammospheres function to determine the stem/progenitor cell phenotype of mammosphere-resident cells. We

further investigated the transcriptional overlap of mammospheres with individual stem cell populations to evaluate the similarity of various tissue compartments to mammary epithelial stem cells cultured *in vitro*. These analyses revealed that 6.52%, 8.35%, and 5.59% of genes (corresponding to 39, 68, and 19 transcripts) common to neural, embryonic, and hematopoietic stem cells, respectively, were found to overlap with genes preferential to mammospheres (Figure 2B-D).

Like mouse mammary epithelial stem cells (Stingl *et al.*, 2006a), hair follicle stem cells can be purified with antibodies to CD49f/integrin-α6 (Blanpain *et al.*, 2004), indicating that these cell populations may share a similar overall expression profile. Indeed, mammospheres shared the highest percentage overlap (14.86% or 11 transcripts) with genes common to hair follicle stem cells reported by two independent investigators (Figure 2E), perhaps reflective of this common phenotype ($CD49f^{high}$). Among the genes common to hair follicle stem cells and mammospheres were stem cell markers CD34 and tenascinC. To support these analyses, we examined the expression of tenascinC protein in mammospheres and differentiated cells (Figure 3) and found that it was expressed in nearly 80% of mammosphere-resident cells while its expression was extinguished in differentiated cells. Thus, in agreement with previous findings that tenascinC is preferentially expressed in populations of stem/progenitor cells from multiple mouse and human tissue compartments (Dontu *et al.*, 2003a; Ivanova *et al.*, 2002; Morris *et al.*, 2004; Ramalho-Santos *et al.*, 2002; Tumbar *et al.*, 2004), we have verified that it is

similarly preferentially expressed in mouse mammospheres, a population of mammary stem/progenitor cells.

Together with the observation that mammospheres contain functional stem cells, these data argue that mammospheres may be employed to investigate regulation of mammary stem cell renewal *in vitro*, analyses that may facilitate the discovery of conserved mechanisms of stem cell regulation from other tissue compartments.

Mammospheres induced to differentiate *in vitro* exhibit evidence of lineage-bias differentiation

We have observed that many markers of stem cells are preferentially expressed in mammospheres and that 85% of the 6305 genes expressed in the adult mammary gland are also expressed by mammospheres induced to differentiate (Figure 1); these data suggest that mechanisms of mammosphere differentiation *in vitro* might mimic normal differentiation of the mammary gland occurring *in vivo*. We found that 8,470 genes were reproducibly expressed in mammospheres induced to differentiate, among which 525 were preferentially expressed in differentiated mammospheres at levels twofold or higher than those in mammosphere cells (Table S2); 250 of these 525 transcripts were reproducibly expressed in the mammary gland (Figure 1C). Moreover, of the 525 genes preferentially expressed in differentiated mammospheres, 186 were not detectably expressed in at least one of the three replicate samples from mammospheres. The 525 genes preferential to mouse mammospheres induced to differentiate included markers of

the myoepithelial lineage such as α-smooth muscle actin, myosin heavy chain myh9, calponin-2, caldesmon-1, and collagen1-α1 (Jones *et al.*, 2004; Lazard *et al.*, 1993). Surprisingly, our microarray data revealed that differentiated mammospheres did not preferentially express any known luminal epithelial-specific genes such as cytokeratins-7, -8, -18, -19, or E-cadherin (Jones *et al.*, 2004; Page *et al.*, 1999; Stingl *et al.*, 2005). However, the observation that mammospheres contain stem cells capable of multi-lineage differentiation *in vivo* excludes the possibility that mammospheres are devoid of luminal progenitors and instead suggests that our methods employed to induce differentiation *in vitro* likely favoured myoepithelial morphogenesis.

Over the course of our studies, investigators (Dontu *et al.*, 2003a) reported the serum-free culture and subsequent induced differentiation of human mammary epithelial cells (human mammospheres). While the conditions used to propagate human and mouse mammospheres were very similar, the culture media used to their differentiation was very different: in these experiments the human differentiation conditions included serum-containing media with subsequent treatment with prolactin, while mouse mammospheres were differentiated in the presence of serum alone.

Since the expression profile of mouse differentiated mammospheres revealed an apparent deficit in luminal differentiation, we wondered whether human mammospheres differentiated in the presence of serum and prolactin preferentially expressed genes characteristic of the luminal lineage. Indeed, by contrast to their mouse counterparts, human differentiated mammospheres expressed a gene signature consistent with a

luminal-epithelial phenotype including the structural filament cytokeratin-18 (O'Hare *et al.*, 1991), luminal fate controller GATA3 (Asselin-Labat *et al.*, 2007; Kouros-Mehr *et al.*, 2006), and luminal-specific adhesion protein E-cadherin (Daniel *et al.*, 1995). Surprisingly, however, none of the common myoepithelial lineage markers were preferentially expressed in human differentiated mammospheres suggesting that mammosphere cells differentiated in the presence or absence of prolactin were different. Accordingly, when we compared genes preferential to both human and mouse cells induced to differentiate (Figure 4) we discovered that human and mouse mammospheres preferentially express few genes in common. Among the 12 transcripts common to human and mouse differentiated mammospheres was the secreted inhibitor of canonical Wnt/β-catenin signaling Sfrp1 (Figure 4B).

An overlap of 37 genes was observed in genes commonly preferential to both human and mouse mammospheres; these transcripts included stemness gene tenascinC and the β-catenin-dependent transcription factor tcf4 (Figure 4A). Interestingly, despite the limited overlap in these studies, genes common to human and mouse mammospheres and differentiated cells support a conserved role for Wnt/β-catenin-dependent regulation of mammary gland development and differentiation (discussed below). Furthermore, considering the intrinsic similarity of their origin tissues, it seems likely that the small degree of overlap of genes commonly preferentially expressed in human and mouse differentiated mammospheres highlights differences between the methods employed to induce differentiation *in vitro*. These findings are exciting as they

argue for the ability to biochemically direct the differentiation of mammospheres along one or another lineage *in vitro*, further extending the utility of mammosphere-based assays in the study of normal mammary gland development.

Evidence for the contrasting functions of canonical and non-canonical Wnt/β-catenin signaling in mammospheres and differentiated mammospheres

Wnt proteins are involved in a complex signaling pathway (Figure 5) that controls stem cell renewal, proliferation, or differentiation of many mammalian tissues (Alonso and Fuchs, 2003; Hari *et al.*, 2002; Ito *et al.*, 2007; Kielman *et al.*, 2002; Kobielak *et al.*, 2007; Korinek *et al.*, 1998; Kubo *et al.*, 2003; Lako *et al.*, 2001; Miyoshi *et al.*, 2002). Furthermore, germline deregulation of the Wnt/β-catenin pathway causes oncogenic transformation in mice (Bruxvoort *et al.*, 2007; Imbert *et al.*, 2001; Michaelson and Leder, 2001; Tsukamoto *et al.*, 1988) while genetic lesions in Wnt pathway members such as Apc are associated with human malignancies (MacLeod *et al.*, 2007; Nojima *et al.*, 2007). For example, mice which express Wnt-1 or β-catenin in their mammary glands under the control of the MMTV promoter exhibit mammary epithelial hyperplasia that eventually progresses to multiple aggressive adenocarcinomas (Imbert *et al.*, 2001; Michaelson and Leder, 2001; Tsukamoto *et al.*, 1988).

To help understand the contribution of Wnt signaling to the transcriptional profile of mammospheres, we examined Wnt pathway components preferentially expressed in mammospheres or their differentiated counterparts (Table 2). Interestingly, genes that

promote the activation of canonical β-catenin signaling were largely expressed in mammospheres, including secreted Wnt-2, β-catenin-dependent transcription factor Tcf4, and the β-catenin shuttle protein Pin1. Similarly, genes that inhibit canonical signaling were expressed preferentially upon differentiation *in vitro*, including secreted frizzled-related proteins Sfrp1 and Sfrp2, and dapper-1, a protein that binds and sequesters disheveled, preventing signal transduction. More importantly, we found preferential expression of non-canonical Wnt5a and receptors frizzled-8 and frizzled-2 in differentiated mammospheres, suggesting that like in other developmental systems, non-canonical Wnt signaling in mammospheres is a driving force during their differentiation. Thus we infer that mammosphere cells are better equipped than their differentiated descendents to transmit canonical Wnt signals. These observations suggest that mammospheres may be ideally suited to study a role during mammary gland differentiation for Wnt signaling, a pathway that is difficult to study *in vivo* due to its complexity of regulation and critical function during embryonic development.

Our observations are further supported by the discovery that many known target genes of canonical Wnt/β-catenin signaling are preferentially expressed in mammospheres (Table 3) (El-Tanani *et al.*, 2004; Howe *et al.*, 2001; Ziegler *et al.*, 2005). Known direct targets of β-catenin-dependent transcription that are preferential to mammospheres include ETS gene PEA3 (38-fold), Cox2/Ptgs2 (37-fold), the runt-related osteoblastic transcription factor Runx2 (6-fold), CyclinD1 (5.6-fold), differentiation fate

regulator Id2 (5.4-fold), osteopontin (2.5-fold), c-jun (2.3-fold), breast cancer stem cell marker CD44 (2.1-fold).

Members of the PEA3 subfamily of Ets transcription factors (PEA3, ER81, and ERM) play important roles in the regulation of mammary epithelial differentiation and stem/progenitor cell fate (Kurpios *et al.*, 2003), are involved in maintenance of the stem cell niche (Chen *et al.*, 2005), and are overexpressed in human and mouse models of breast cancer (Shepherd *et al.*, 2001; Trimble *et al.*, 1993). The founding member of this subfamily, PEA3 (Etv4), was cloned in our lab (Xin *et al.*, 1992), and is of particular interest in mammary gland development and oncogenesis (Kurpios, 2005). Furthermore, PEA3 cooperates with β-catenin in the transcription of multiple Wnt target genes (Crawford *et al.*, 2001; El-Tanani *et al.*, 2004; Howe *et al.*, 2001), some of which were preferentially expressed in mammospheres such as osteopontin (OPN/Spp1), and Cox2/Ptgs2 (Table A3). Moreover, several other known β-catenin target genes that are also preferential to mammospheres contain uncharacterized PEA3 binding sites (Xin *et al.*, 1992) upstream of their transcription start site (Ziegler *et al.*, 2005), suggesting that PEA3 may additionally cooperate with β-catenin at these promoters. Altogether, the 38-fold preferential expression of PEA3 mRNA in mammospheres suggested that, perhaps in cooperation with β-catenin, this gene may contribute significantly to the overall phenotype of mammospheres, a hypothesis consistent with its observed role in mouse mammary stem cell regulation *in vivo* (Kurpios, 2005).

To validate these observations we confirmed by qRT-PCR the differential expression of PEA3 and its subfamily members ER81 and ERM (Figure 6), demonstrating that all three transcripts are highly preferentially expressed in mammospheres compared to differentiated cells. PEA3 expression was 38.97 and 38.5-fold higher in mammospheres detected by microarray and RT-PCR, respectively. Moreover, when assessed by immunocytochemical analyses, PEA3 protein was detected in 75% of mammosphere cells and in 23% of differentiated mammosphere-derived cells (Figure 6). Our microarray analyses revealed that ER81 and ERM were expressed preferentially in mammospheres by 2.53 and 3.58-fold, respectively and were downregulated upon differentiation; qRT-PCR analyses confirmed their differential expression in mammospheres and showed that their expression was extinguished upon differentiation (Figure 6C). Together these analyses demonstrate the preferential expression of PEA3 subfamily members in mammosphere-derived cells, confirming previous observations that these transcription factors contribute to mammary epithelial stem/progenitor cell phenotype *in vivo*.

Transcriptional profile of mouse mammary tumour-initiating cells cultured *in vitro*

Mammary gland-specific expression of the rat Neu cDNA is achieved under the control of the mouse mammary tumour virus (MMTV) viral promoter; this germline expression of Neu induces, with median latency of 315 days, metastatic disease resembling human c-erbB2-overexpressing breast cancer (Guy *et al.*, 1992; Shepherd *et*

al., 2001). Based on our findings that mammospheres afford a means to investigate *in vitro* the molecular regulation of stem cell renewal, we attempted to culture cells *in vitro* from primary mouse mammary tumours induced by the MMTV-Her2/Neu transgene (Figure 7). In a process analogous to that used to derive mouse mammospheres, suspensions of primary Neu-induced tumour cells cultured in stem cell media give rise to mouse mammary tumourspheres after 7 days (Kurpios, 2005). Tumoursphere suspension cells are passaged subsequently every 4 days by mechanical dissociation, and can exist in culture for indefinite periods under this regime. Importantly, a high percentage of tumoursphere-resident cells (25-40%) seed the growth of secondary tumours upon orthotopic or subcutaneous engraftment in syngeneic recipient mice (Kurpios, 2005), demonstrating the utility of this system to propagate functional mammary tumour-initiating cells over multiple serial passages *in vitro*. Furthermore, these secondary tumours can be retransplanted *in vivo* for many successive generations highlighting the seemingly unlimited potential of tumoursphere-resident cells for proliferation and self-renewal (Personal communication: Kurpios and Hassell, 2007). Thus tumourspheres contain functional cancer stem cells and offer a renewable source of such cells for molecular studies.

With the knowledge that tumourspheres contain a high fraction of tumour-initiating cells (candidate cancer stem cells), we were eager to learn whether their transcriptional profile lends support to the hypothesis that cancer arises in stem cells and whether cancer stem cells maintain much of the molecular phenotype of normal tissue

stem cells. To this end, three biologically distinct tumoursphere preparations were used to assemble a representative gene expression profile of tumourspheres cultured *in vitro*, using conditions already reported for mammosphere transcriptional profiling. We found that tumourspheres expressed 9,592 of 22,690 total transcripts on the MOE430A array, judged by present calls in each of biological 3 samples. Tumourspheres preferentially expressed only 280 genes versus mammospheres (Table S3), where 181 of these transcripts were assigned an absent call in at least one mammosphere sample. A similar comparison revealed that tumourspheres preferentially expressed 607 genes compared to mammospheres induced to differentiate (Table S4); of these genes 370 were assigned an absent call in at least one sample from differentiated mammospheres. Further analyses revealed that tumourspheres more closely resemble the transcriptional profile of mammospheres than differentiated cells; 373 genes were preferentially expressed in both tumourspheres and mammospheres (Figure 8A), while only 48 transcripts were preferentially expressed in both tumourspheres and differentiated mammosphere cells (Figure 8B).

Interestingly, tumourspheres preferentially expressed genes known to function in mammary stem cell regulation or that have been previously used to enrich stem cells from other tissues or tumours. Included among these genes are CD49f/α6-integrin (Stingl *et al.*, 2006a), human neural/brain tumour stem cell marker CD133/prominin1 (Singh *et al.*, 2003; Singh *et al.*, 2004b), and the regulators of mammary stem cell fate Notch1 and its ligand Delta-like 1 (Dontu *et al.*, 2004). Moreover, similar to our findings with

mammospheres, the expression profile of tumourspheres was most similar to that of purified hair follicle stem cells than to any other stem cell population we analyzed, including neural, hematopoietic, and embryonic (data not shown). However, we discovered that tumourspheres preferentially express more genes in common with hair follicle stem cells than do mammospheres (Figure 8C). This may suggest that the stem cell transcriptional phenotype is further amplified in mouse tumourspheres, in accordance with previous observations from our lab that tumourspheres contain a higher fraction of functional stem cells than do mammospheres (Kurpios, 2005). Thus our gene expression analyses are consistent with the hypothesis that tumourspheres cultured *in vitro* contain a high fraction of cancer stem cells.

Recent studies identified CD44 and CD24 as important markers of prospective human breast cancer stem cells (Al-Hajj *et al.*, 2003). These authors showed that primary tumour cells with surface phenotype $CD44^{+}/CD24^{-}$ could be isolated from the bulk population and could exclusively seed the growth of a new tumour following xenograft transplants into NOD/SCID mice. Importantly, CD44 is expressed at higher levels in mouse tumourspheres compared with mammospheres (1.9 fold) or differentiated mammospheres (4.1 fold), and thus we speculate that the high fraction of tumour-initiating cells in mouse tumourspheres represents a rich population of true cancer stem cells.

To further evaluate the transcriptional profile of tumoursphere-resident cells, we compared tumoursphere-enriched genes to the expression profiles of normal and tumour

$CD44^+/CD24^-$ and $CD24^+/CD44^-$ cell populations reported elsewhere (Shipitsin *et al.*, 2007). In human breast tumours $CD44^+/CD24^-$ cells are responsible for the transfer of disease to NOD/SCID mice (Al-Hajj *et al.*, 2003), and are prospective cancer stem cells. Indeed, the authors reported that these cells express many known markers of stem cells and thus display an overall transcriptional profile consistent with a stem cell identity (Shipitsin *et al.*, 2007). We discovered that 46 transcripts (7.4%) preferential to tumourspheres overlapped with normal $CD44^+/CD24^-$ breast cells (Figure 9A), while 28 transcripts (4.5%) overlapped with normal $CD24^+/CD44^-$ cells (Figure 9B). Thus tumourspheres share larger overlap with normal $CD44^+/CD24^-$ cells, a population enriched for stem cell markers. The same was true for comparisons of tumourspheres with tumour $CD44^+/CD24^-$ and $CD24^+/CD44^-$ cells. Tumourspheres expressed 86 transcripts (13.8%) in common with cancer $CD44^+/CD24^-$ cells (Figure 9C) and 61 transcripts (9.8%) in common with cancer $CD24^+/CD44^-$ cells (Figure 9D). Together, these comparisons show that tumourspheres preferentially express many genes from a signature defining prospective breast cancer stem cells, further supporting the notion that tumour-initiating cells within tumourspheres are cancer stem cells propagated *in vitro*.

DISCUSSION

Mammospheres are a versatile system to study stem cell process

We have demonstrated a significant overlap of mammosphere-enriched genes with those globally expressed by other stem cell populations (Figure 2) and were

intrigued to learn that mammospheres shared the most significant overlap with hair follicle stem cells, a population similarly marked by the expression of CD49f/integrin-α6. It is therefore a possibility that mammary and hair follicle stem cells are functionally related and may share mechanisms that maintain their undifferentiated state. Indeed, both the mammary epithelia and hair follicle tissues are derived from ectoderm, and fetal mammary epithelium has rudimentary plasticity to differentiate into salivary tissue, another ectodermally-derived compartment (Kratochwil, 1969). Thus the mammary gland transplant assay may help to determine the plasticity of hair follicle stem cells, by assessing their potential to transdifferentiate upon exposure to the mammary stromal environment. Furthermore, the plasticity of mammospheres may be assessed by exposure of these cells to embryonic tissue compartments in experiments similar to those previously described with fetal epithelia (Kratochwil, 1969; Sakakura *et al.*, 1987).

Mammospheres as a model to explore the molecular control of mammary gland development

Consistent with our transcriptional profiling data which demonstrate that mammospheres preferentially express genes characteristic of stem/progenitor cell populations, results from our laboratory show that mammospheres contain *bone fide* stem cells capable of extensive self-renewal and differentiation along both the myoepithelial and luminal lineages upon transplant *in vivo* (Kurpios, 2005). We were therefore surprised to learn that upon differentiation *in vitro*, genes characteristic of

luminal epithelial cells were not preferentially expressed in differentiated mammospheres (Stingl *et al.*, 2006b). For example, whereas markers of the myoepithelial lineage such as α-smooth muscle actin were very abundant upon *in vitro* differentiation of mammospheres, luminal lineage markers such as CK8, CK18, E-cadherin, and MUC-1 were not preferentially expressed in these cells. Collectively, these data may suggest that whereas mammospheres are capable of complete lineage differentiation *in vivo*, the methods employed to induce their *in vitro* differentiation favoured myoepithelial morphogenesis at the expense of luminal cells. These observations further suggest that mouse mammospheres may be useful as a means to explore and identify factors directing mammary differentiation along specific lineages.

Conversely, transcriptional profiling of human differentiated mammospheres reveals the expression of numerous transcripts characteristic of the mammary luminal lineage (Dontu *et al.*, 2003a). Interestingly, these cell populations fail to express the myoepithelial lineage marker α-smooth muscle actin. These data clearly contrast the differentiation of mouse mammospheres from our laboratory and further suggest differences in the methods employed to induce differentiation of mouse and human mammospheres *in vitro*. We speculate that in these human studies the addition of prolactin, a lactogenic hormone shown previously to induce functional and terminal differentiation along the luminal and alveolar/secretory lineages (Horseman, 1999; Shamay *et al.*, 1989; Silberstein, 2001; Stelwagen *et al.*, 1999), induces a strong luminal phenotype in human mammospheres at the expense of myoepithelial lineage

differentiation. In this regard, mouse mammospheres may prove to be a highly useful tool to explore the molecular control of normal mammary gland differentiation. Thus, future studies should employ mouse mammospheres to analyze the role of prolactin and other growth factors during mouse mammary epithelial lineage differentiation.

Mammospheres as a model to study signal transduction pathways directing mammary gland development

CANONICAL WNT/β-CATENIN SIGNALING IN MAMMOSPHERES

Compared to their differentiated descendants, mammospheres preferentially expressed transcripts suggestive of canonical Wnt activation, including both Wnt pathway members (Table 2) and target genes (Table 3). For example, we found that mammospheres differentially expressed Tcf4, a β-catenin-dependent transcription factor, Wnt2, a secreted activator of canonical signaling, and Pin1, a prolyl-isomerase that stabilizes β-catenin. β-catenin-knockout mouse embryos exhibit failed gastrulation because of a defective embryonic ectoderm (Haegel *et al.*, 1995; Huelsken *et al.*, 2000). Tcf4-knockout mice die at birth, and exhibit depletion of intestinal epithelial stem cells (Korinek *et al.*, 1998). Thus the Wnt/β-catenin machinery expressed preferentially in mammospheres are required for proper embryonic development of epithelial compartments (the embryonic lethality of these mutations precludes complete investigation of their effect on mammary development). Pin1-knockout mice show a proliferation defect during pregnancy resembling that of CyclinD1 knockout mice (Liou *et*

al., 2002), accompanied by substantially reduced levels and altered subcellular localization of β-catenin protein through a direct interaction (Ryo *et al.*, 2001). In addition to promoting β-catenin-dependent CyclinD1 transcription, Pin1 also binds CyclinD1 and induces its nuclear localization in a positive-feedback loop. These data implicate Pin1 in promoting β-catenin-induced cell proliferation. Since Pin1 is a direct E2F-family target gene (Ryo *et al.*, 2002), it was exciting to learn that E2F5, Pin1 and CyclinD1 are all preferential to mammospheres by twofold or greater, supporting our claim that canonical β-catenin-dependent transcription is active in mammospheres. Other targets of canonical β-catenin signaling are differentially expressed in mammospheres, including cell surface glycoprotein CD44 and one of its binding partners, osteopontin (OPN). The interaction of CD44 and osteopontin induces cell survival of normal and metastatic cells through a mechanism involving PI3K/Akt activation (Lin and Yang-Yen, 2001), and functions in both tissue repair (Liaw *et al.*, 1998) and tumor cell survival (Crawford *et al.*, 1998). Thus it is possible to envision a role for CD44 and osteopontin in the regulation of PI3K/Akt signaling in mammospheres downstream of Wnt, and that together these genes may contribute to the survival or maintenance of mammary epithelial stem cells *in vitro*. Supporting this idea is the observation that Pik3r1, a regulatory subunit of PI3-kinase with a potent negative effect on Akt posphorylation and PI3K activity (Ueki *et al.*, 2000), is highly upregulated upon differentiation of mammospheres *in vitro*. Perhaps more importantly, osteopontin has been identified as a critical regulator of the hematopoietic stem cell niche (Calvi *et al.*, 2003; Nilsson *et al.*,

2005) and negatively regulates the size of the population by suppressing stem cell proliferation (Stier *et al.*, 2005). These reports also show that osteopontin expression determines the size of the niche and the localization of bone marrow stem cells *in vivo* in a direct interaction with the surface of stem cells via β1-integrin (Nilsson *et al.*, 2005). This molecular interaction warrants further investigation in mammospheres, as mammary stem cells can be purified from bulk primary mammary eptihelia with antibodies to β1-integrin (Shackleton *et al.*, 2006). Furthermore, since CD44 marks human breast cancer stem cells (Al-Hajj *et al.*, 2003) and has roles in both cell adhesion (hyaluronan receptor) and PI3K/Akt regulation via its interaction with osteopontin, investigations of the loss-of-function phenotypes for these proteins in mammospheres, as well as the transcriptional profile of $CD44^+$ mammosphere-resident cells, will likely prove informative.

Based on transcriptional profiling, we have proposed that canonical β-catenin-dependent transcription is active in mammosphere cells. To confirm this further future experiments should make use of reporter systems such as TOPFLASH or TOPeGFP (Kim *et al.*, 2000; Moriyama *et al.*, 2007) to investigate the status of β-catenin-dependent transcription in mammospheres and/or tumourspheres and their descendant cells. Furthermore, we reported that some of the Wnt/β-catenin target genes expressed preferentially in mammosphere-resident cells (Table A3) have PEA3 binding sites within their promoter regions (a non-exhaustive list). Considering the highly differential expression of PEA3, and to a lesser extent of ER81 and ERM, it seems likely that PEA3 subfamily members perform important functions in determining the transcriptional profile

of mammospheres, and may therefore direct aspects of mammary epithelial stem cell function as suggested previously (Kurpios, 2005). The combined employment of the mammosphere system, lentiviral vectors, and dominant-negative forms of PEA3 available in our lab may prove useful to address these questions in future experiments.

NON-CANONICAL WNTS DURING DIFFERENTIATION

Mammospheres induced to differentiate preferentially express genes requisite for non-canonical Wnt signaling such as Wnt5a and Frizzled8, suggesting that non-canonical signaling may be a feature of differentiated cells. In support of this hypothesis, rat Neural stem cells express canonical Wnt2 (reported here as preferential to mammospheres) while the same cells induced to differentiate express increasing amounts of non-canonical Wnt5a and decreasing amounts of β-catenin (Lange *et al.*, 2006). These observations indicate a diminished role for β-catenin signaling during differentiation and a corresponding increase in non-canonical Wnt signaling. Likewise, Wnt5a protects differentiated osteoblasts from apoptosis in a β-catenin-independent but Akt-, PI3K-, and ERK-dependent fashion supporting a role for non-canonical signaling in the survival or identity of differentiated cells. Moreover, Wnt5a induces proliferation and survival of human vascular endothelial cells, a phenotype reversed by exogenous delivery of soluble Frizzled4 extracellular domain (Masckauchan *et al.*, 2006). These data argue for contrasting functions of canonical and non-canonical Wnt signaling during differentiation. Perhaps more importantly, non-canonical Wnts override canonical signaling throughout

vertebrate biology; for example, the non-canonical Wnt5a can antagonize secondary axis formation induced by exogenous canonical Wnt1 (Torres *et al.*, 1996). However, this antagonistic effect of Wnt5a is a direct effect (extracellular), as Wnt5a was unable to antagonize axis formation upon overexpression of β-catenin or dominant negative GSK3β (see Figure 5). Further suggesting a powerful role for Wnt5a in differentiation, antisense-mediated loss of Wnt5a induces transformation of mammary epithelial cells and mimicry of the Wnt1-transformed phenotype (Olson and Gibo, 1998), while exogenous Wnt5a reverts transformation and induces differentiation of both renal cell carcinoma (Olson *et al.*, 1998) and uroepithelial tumour cells (Olson *et al.*, 1997). Together these findings corroborate an integrated role for non-canonical Wnt/β-catenin signaling in the induction of mammary epithelial differentiation *in vitro*.

We propose that non-canonical signaling may be a decisive factor in mammary epithelial stem cell differentiation *in vitro;* to address this question, the effect of exogenous Wnt5a or other related non-canonical Wnt proteins should be established in cultured mammospheres. While sphere formation may not be an optimal assay *per se* for mammary stem cells *in vitro*, differentiating mammosphere-derived cells adhere to the culture vessel and can form morphologically-discernable colonies (Kurpios, 2005), affording a rudimentary assessment of morphological differentiation. However, the functional differentiation of mammary epithelial cells *in vitro* is perhaps best exemplified by the formation of polarized epithelia and the secretion of milk protein after stimulation with lactogenic hormones (Horseman, 1999). Others have cultured mammary epithelial

cells in collagen (Montesano and Soulie, 2002) or basement membrane-type gels (Dontu *et al.*, 2003a) and have assessed fundamental luminal morphogenesis, polarization, and casein expression following the addition of agents to induce differentiation. To augment monolayer clonogenic assays, it may be possible to employ mouse mammospheres under similar conditions, assaying the potential of exogenous Wnts or soluble frizzled proteins to induce the functional differentiation of mammospheres in serum-free 3D culture along one or both lineages. Also, as suggested by the apparent activation of canonical Wnt signaling in mammospheres, it may be possible to revert the phenotype induced by exogenous non-canonical Wnt5a, with canonical Wnt/β-catenin agonists such as β-catenin or dominant-negative GSK3β.

NOTCH SIGNALING IN MAMMARY EPITHELIAL CELL IDENTITY

While we have focused here on Wnt/β-catenin signaling, the interpretation of mammosphere transcriptional profiles should be extended to other developmental transduction pathways in future experiments. For example, the preferential expression of Notch and its Delta-like ligand in mammospheres, and even higher expression in tumourspheres, argues that these proteins may play a role in the self-renewal of normal and transformed mammary stem/progenitor cells *in vitro*. Notch, β1-integrin, and EGFR physically interact in neurospheres (Campos *et al.*, 2006), potentiating EGFR activity and stimulating growth, while the interaction and coregulation of EGFR and β1-integrin has been reported in 3D culture of mammary epithelial cells (Wang *et al.*, 1998). Our data

prompt questions about these interactions in mammospheres, with emphasis on the recent discovery (Shackleton *et al.*, 2006) that β1-integrin highly enriches for mammary epithelial stem cells, a Notch-overexpressing and EGF-responsive cell population *in vitro*. Since the preferential expression of classical Notch target genes such as hairy and enhancer-of-split (Hes1) is not observed in mammospheres compared to cells differentiated *in vitro*, investigation of Notch signaling in mammospheres is all the more intriguing. Indeed, preliminary experiments are already underway in our laboratory to learn the effects of Notch disruption in these cells (Barcelon, 2006). Moreover, Notch components are highly preferentially expressed in tumourspheres, confirming observations that deregulation of Notch signaling supports the oncogenic transformation of mammary epithelia (Callahan and Egan, 2004; Hu *et al.*, 2006). Thus similar experiments with tumourspheres are in progress to learn if Notch signaling inhibitors can attenuate cancer stem cell self-renewal *in vitro* and *in vivo*. While our previous approach involved the disruption of Notch signaling in tumourspheres and mammospheres through small molecule γ-secretase inhibitors, developmental signaling pathways like Notch and Wnt depend on cell-cell and cell-matrix contact and can also therefore be modulated easily by supplementing culture media with purified ligands or peptides. This latter approach may facilitate the discovery of more specific and direct roles for Notch and other signaling pathways in the regulation of stem cell maintenance or differentiation. Though speculative, this scenario provides a framework to use the mammosphere/tumoursphere culture system with functional genomics and dissect *in vitro*

the molecular basis of mammary epithelial differentiation and its deregulation during carcinogenesis.

CROSSTALK AMONG SIGNALING PATHWAYS REGULATES MAMMARY DEVELOPMENT AND DIFFERENTIATION

While Notch and β-catenin signaling are attractive candidates for the regulation of mammary stem cell renewal, crosstalk of these with other cellular signaling pathways play significantly in mammary gland development. Mammospheres are no exception: preferential expression in these cells of molecular equipment and target genes from signaling pathways like TGFβ, BMP, PI3K/Akt, and Vitamin A (retinoic acid) confirm extensive findings that these molecules regulate mammary epithelial proliferation, development, survival, and differentiation (Daniel *et al.*, 2001; Hens *et al.*, 2007; Hutchinson *et al.*, 2001; Petersen *et al.*, 1998). Hence we believe mammospheres will offer valuable opportunities to elucidate roles for these pathways in mammary gland development.

The runt-related transcription factor Runx2 directs crosstalk between pathways such as BMP, PI3K, and TGFβ. For example, TGFβ-1 and BMP-2 (both highly preferential to mammospheres) cooperate to induce Runx2 expression and that of its target genes (Lee *et al.*, 2000). Runx2 is also downstream of Wnt signaling and is preferentially expressed in mammospheres by six fold, suggesting a substantial role for its target genes in defining the mammosphere phenotype. Runx2 expression in embryonic development

is highly restricted to the early mammary gland, primordial bone, and the genital tubercle (Otto *et al.*, 1997), supporting the theory that the observed preferential expression of Runx2 in mammospheres reflects events that are critical to mammary gland development. Moreover, Runx2-knockout mice die post-natally and exhibit failed bone formation (Komori *et al.*, 1997; Otto *et al.*, 1997); intriguingly, this is due to the failed maturation of osteoblasts, which form bones and the hematopoietic stem cell niche. In mammary epithelial cells, Runx2 directly activates the transcription of osteopontin (Inman and Shore, 2003), the osteoblastic and mammosphere-enriched gene that defines the bone marrow stem cell niche and may play a similar role in the mammary gland. This notion is supported by several observations: the highly restricted embryonic expression of Runx2 suggesting indispensable function in mammary development; the interaction of its transcriptional target, osteopontin, with stem cell markers such as CD44 and β1-integrin (discussed previously); and the severe hematopoietic impairment of osteoblast-deficient transgenic mice (Visnjic *et al.*, 2004). Thus in mammospheres, Runx2 may ultimately induce the mechanical and chemical coupling of stem cells to more differentiated progeny, preserving their undifferentiated state and forming a niche akin to that formed by osteoblasts to support hematopoietic stem cells. Supporting this concept are our observations that mammospheres contain a high fraction of myoepithelial progenitors (Kurpios, 2005), a phenomenon that is consistent with the attachment of stem cells to the myoepithelial compartment *in vivo* (Asselin-Labat *et al.*, 2006). In this scenario, perhaps production of daughter progenitor cells from a stem cell

continues until a threshold is reached, when more committed progeny feed back on the stem cell to inhibit its proliferation; if true, factors such as Runx2, osteopontin, CD44 and β1-integrin may participate in this mechanism. Other genes downstream of Runx2 (Vaes *et al.*, 2006) are also differentially regulated in mammospheres or mammospheres induced to differentiate including matrix metalloproteinase 9, mast cell proteases 6 and 7, delta-like 1 (notch ligand), c-fos-induced growth factor, and kit ligand. Since osteopontin can negatively regulate stem cell proliferation, blocking antibodies or shRNAs to osteopontin may induce mammosphere and tumoursphere-resident stem cells to proliferate, a relatively straightforward method to investigate the function of osteopontin in mammary epithelial stem cell status.

Further evidence supports a role for Runx2 target gene osteopontin, perhaps via niche initiation, as a facilitator of lymph node metastases (Allan *et al.*, 2006); such activities are attributed to transformed stem cells (Li *et al.*, 2007b) by many proponents of the cancer stem cell hypothesis. Thus the exploration of the Runx2-deficent and related phenotypes in mammospheres and tumourspheres may reveal a larger signaling network that functions in stem cell renewal, interactions with a niche, adhesion, and metastasis.

Finally, we have identified one other interesting expression pattern within mammospheres related to retinoic acid signaling. Though retinoic acid is traditionally associated with differentiation, we were intrigued to learn that compared to differentiated cells, mammospheres express higher levels of retinoic acid receptor beta, retinol binding protein (produces retinoic acid), cellular retinoic acid binding protein (a

competitive inhibitor of signaling) and three retinoic acid-induced transcripts Raet1c, Stra13, and Rai14; this signature is similarly amplified in tumourspheres by comparison to differentiated cells. However, in mammary epithelial cells, attenuated retinoic acid signaling induces proliferation and abrogates response of these cells to interactions with the extracellular matrix (Seewaldt *et al.*, 1997). Indeed, these are two facets common to stem cell maintenance and terminal differentiation as both cell types are non-proliferative and have phenotypes determined by cell-matrix and cell-cell contacts. Thus future analyses should investigate the distribution of various components of retinoic acid signaling in mammospheres, differentiated cells, and tumourspheres to provide more insight into this seemingly paradoxical mammosphere-restricted activity of retinoic acid signaling.

A sphere-based culture model to investigate cancer stem cell biology *in vitro*

Independent reports of human tumour comparments have prompted significant interest in the possibility that normal stem cells and their tumourigenic colleagues, cancer stem cells, share a pedigree and have a multitude of properties in common (Tan *et al.*, 2006; Wang and Dick, 2005). Since the first identification of prospective leukemic stem cells (Lapidot *et al.*, 1994), tumour-initiating cells have been identified for an array of human cancers based on differences in surface phenotype among the bulk tumour cells. In these studies, presumptive markers of various adult stem cells are used to purify rare cellular subfractions which are subsequently shown to be highly enriched for tumour-

initiating cells when transferred as xenografts into NOD-SCID mice (Al-Hajj *et al.*, 2003; Collins *et al.*, 2005; Kim *et al.*, 2005; Li *et al.*, 2007a; Prince *et al.*, 2007; Singh *et al.*, 2003). These subfractions are comprised of cells that share characteristics with stem cells and exclusively contain the cells capable of regenerating a tumour in a new animal, suggesting that this enriched population of tumour-initiating cells is composed of cancer stem cells, at least in part.

Thus the cancer stem cell hypothesis is a convenient model to explain global observations about tumour cell heterogeneity and functional evidence for tumour-initiating cells. Furthermore, it is well established that poorly differentiated tumours of many human tissues are correlated with more aggressive or invasive behaviour and poor clinical outcome; indeed tumour grade is established partly from status of differentiation. The existence of a cancer stem cell is also an attractive scenario to account for these clinical data. Poorly differentiated tumours may contain a higher fraction of transformed cancer stem cells, which like their normal stem cell ancestors bear unlimited potential to proliferate and differentiate. However, unlike their normal counterparts, cancer stem cells within poorly differentiated tumours may suffer deregulated control of symmetric vs. asymmetric division, accounting for their increased incidence within the bulk tumour population. These observations argue that cancer stem cells are functionally discernable by their surface phenotypes and therefore they bear important ramifications for the design of durable therapeutics; perhaps this is why a vast majority of poorly-differentiated tumours relapse following conventional therapies.

Mouse Her2/Neu-induced tumourspheres express CD44 and CD24 as judged by microarray analyses and immunocytochemistry (Personal communication: Kurpios and Hassell, 2007), while human breast cancer stem cells likely have a $CD44^+/CD24^-$ phenotype (Al-Hajj *et al.*, 2003). Our analyses with tumourspheres agree with previous observations that prospective breast cancer stem cells ($CD44^+/CD24^-$ breast tumour cells) express many classical markers of stem cells by comparison to the $CD24^+/CD44^-$ population, which bear strong transcriptional resemblance to luminal epithelial cells (Shipitsin *et al.*, 2007). These data seem to support the adult stem cell as the place of origin for tumourigenesis, though direct biological evidence for the transformation of normal stem cells remains elusive. Interestingly, the authors also demonstrated that CD24 was highly upregulated in distant metastases compared to primary tumours (Shipitsin *et al.*, 2007). This apparent shift in phenotype is problematic, since normal mammary epithelial stem cells appear to have a $CD24^{low/-}$ phenotype (Shackleton *et al.*, 2006; Stingl *et al.*, 2006a); if normal stem cells are more similar to primary human breast tumour cells than to distant metastases, the question arises whether cancer stem cells are transformed mammary stem cells and whether these cells are in fact responsible for metastatic initiation (Li *et al.*, 2007b). However, the authors also noted that in some primary tumours, $CD24^+/CD44^-$ cells harboured additional genetic rearrangement compared to $CD44^+/CD24^-$ cells. These data argue for a clonal evolution mechanism among cells lower in the hierarchy than breast cancer stem cells, an event that might be correlated with an increase in CD24 expression and higher metastatic capacity. Future

experiments should address the relative potential of $CD44^+/CD24^-$ and $CD24^+/CD44^-$ cells in tumourspheres to engraft *in vivo*. Though we have already established a high, unprecedented fraction (25-40%) of functional tumour-initiating cells within mouse tumourspheres (Kurpios, 2005), sorted populations of $CD44^+/CD24^-$ or $CD24^+/CD44^-$ cells may be further enriched for tumour-initiating cells assessed by allograft transplant studies, a scenario that would confirm data pertaining to human cell populations of similar phenotype (Al-Hajj *et al.*, 2003; Shipitsin *et al.*, 2007). Also, considering that Her2/Neu-induced mouse tumours give rise to metastases with high frequency (Guy *et al.*, 1992), any difference in metastatic potential between $CD44^+/CD24^-$ and $CD24^+/CD44^-$ cells should be assessed using *in vitro* measures of invasive potential and *in vivo* propensity to metastasize preferably to the lungs as described previously (Guy *et al.*, 1992). These experiments will help to challenge the hypothesis that tumour-initiating cells in tumourspheres are cancer stem cells, and whether they bear clinical relevance to metastatic progression *in vivo*.

CHAPTER 2

A FUNCTIONAL GENOMICS APPROACH TO IDENTIFY NOVEL THERAPEUTIC TARGETS OF MAMMARY TUMOUR-INITIATING CELLS

INTRODUCTION

In pursuit of novel breast cancer therapies: functional genomics applied to cancer stem cells

Global gene expression profiling - the process of enumerating the set of genes whose transcription is active in a given cell population – has become a powerful biological tool and has spawned novel fields of study such as bioinformatics and functional genomics. Microarray expression/transcriptional profiling (Schena *et al.*, 1995) has been used to identify the molecular character of countless cell types and to study, on a whole-genome basis, the effect of perturbagens on the transcription of known and novel target genes. Applied to breast cancer in humans, transcriptional profiling has been employed to generate tumour gene signatures that correlate with parameters of disease such as disease-free survival, metastases, and failure of therapy (Dressman *et al.*, 2006; Glinsky *et al.*, 2005; Nuyten *et al.*, 2006; Sotiriou *et al.*, 2003; Wang *et al.*, 2005; West *et al.*, 2001). These gene signatures may be powerful predictors of disease outcome and therefore have important prognostic value. Similar gene signatures from mouse models of human malignancy can be used to find candidate biomarkers of human disease, and to test the potential of these markers as therapeutic targets in functional studies (functional genomics). Here we report that mouse Her2/Neu-induced tumourspheres may provide an *in vitro* means to find candidate genes involved in tumour initiation or progression *in vivo*.

RNAi – a global tool to evaluate gene knockdown

RNA interference (RNAi) has recently emerged as a powerful and versatile tool to approximate loss-of-function phenotypes in vertebrates. The RNAi machinery derives from an intrinsic cellular response to infection with double-stranded RNA viruses (Hu *et al.*, 2002), and offers a simple method to inhibit the expression of any gene at the level of its messenger RNA transcript. Our functional genomics approach involves the identification of candidate genes in tumourspheres followed by the knockdown of these genes in RNAi studies; the delivery of short hairpin RNAs (shRNAs) to induce RNAi silencing can be mediated by retroviral infection or nucleic acid transfection. Our overall objective is to screen for genes with roles in tumour cell viability, metastatic potential, or other measurable parameter of tumour progression *in vitro*.

To this end, we have defined a molecular signature for cancer stem cells cultured *in vitro* using transcriptional profiling, with the intention to systematically assess the requirement of these genes in the tumour phenotype. We report a proof-of-principle experiment designed to demonstrate the utility of tumourspheres in such a system and have contributed validated shRNAs as the first of two steps in this approach.

RESULTS

A molecular signature for mouse breast cancer stem cells

To establish a cohort of candidate genes as biomarkers or therapeutic targets of mouse mammary cancer stem cells, we first enumerated genes preferential to tumourspheres compared to mammospheres induced to differentiate. These genes highlight observed biological differences between the cell populations, and include markers of proliferation, stem/progenitor cells, and Her2/Neu-induced mammary tumours in general. This list of genes was then subjected to hierarchical clustering (Figure 10), revealing genes whose expression is similar in both mammospheres and differentiated cells, but different in tumourspheres. Our intention was to eliminate more obvious genes associated with proliferation or the mammary epithelial phenotype, independent of the status of normal differentiation. The resulting set of genes was finally overlapped with those preferentially expressed in primary Her2/Neu-induced mouse mammary tumours analyzed in our lab, helping to ensure their relevance to *in vivo* tumour progression (Figure 10A). A final list of these *tumourness* genes is shown in Table S5, and their expression levels across each of the samples analyzed is profiled in Figure 10C. Among the genes on this list are lipocalin2, aldolase 3C, tight junction claudins 3, 4, and 7, kappa-casein, Iroquois-related homeobox-3, and thrombospondin-1. Furthermore, cytokeratin-18 and -8 are tumourness genes, supporting observations throughout the literature that Her2/Neu-induced breast tumours are mostly luminal in origin

(Herschkowitz *et al.*, 2007; Hoadley *et al.*, 2007) and express proteins characteristic of luminal epithelial cells (Jones *et al.*, 2004).

Expression of tumourness genes in mouse mammary tumours

An ideal candidate *tumourness* gene would be expressed in tumour cells independent of external factors, exemplifying a role for such a gene in the progression of tumour growth, survival, or subsequent metastasis. To confirm that the transcriptional profile of selected *tumourness* genes was echoed by protein expression *in vitro* and from primary tumours *in vivo*, the expression of lipocalin 2, and tight junction proteins claudin-3 and claudin-7 was assessed by antibody staining (Figure 11) in cultured tumoursphere line #3736, and in paraffin sections of primary tumour #3691 and secondary engrafted tumour #3852. Our preliminary data reveal that the expression and subcellular localization of these three genes remains unchanged across three sample types, supporting the use of our tumoursphere model to define a molecular signature of tumour-specific genes from which candidates can be chosen for functional studies.

Derivation of Neu-overexpressing cell line HTN

Our functional genomics approach consists of transcriptional profiling to identify genes which may be required for the viability of tumour-initiating cells. We intend to apply these genes systematically in short-hairpin (shRNA)-mediated screens to learn of their potential role in tumoursphere self-renewal or propagation *in vitro*. Based on

evidence that tumour cell viability is dependent on Neu protein expression in conditional mouse models (Moody *et al.*, 2002), human disease (Baselga *et al.*, 1996; Baselga *et al.*, 1999; Cobleigh *et al.*, 1999; Pegram *et al.*, 1998; Slamon *et al.*, 2001; Vogel *et al.*, 2002), and in human erbB2-positive breast cancer cell lines (Choudhury *et al.*, 2004; Faltus *et al.*, 2004), we devised a proof-of-principle experiment to employ shRNAs in the knockdown of rat Neu in tumoursphere cells. While the most basic formulation of this experiment assumes that all cells within tumourspheres express Neu and remain dependent on its expression for viability, the observation that human erbB2-positive and Neu-induced mouse mammary tumours can relapse in the absence of Neu signaling (Hortobagyi, 2001; Moody *et al.*, 2002) prompted us to evaluate the expression of the transgene in tumourspheres. As demonstrated in Figure 12, the high ubiquitous expression (Figure 12A) and activation of rat Neu protein (phosphorylated Neu, Figure 12B) in tumoursphere line 3736 suggests the plausibility of our proof-of-principle experiment to compromise Neu expression in these cells.

De novo design of short hairpin sequences requires validation *in vitro*, and to this end we derived a stable adherent cell line with constitutive expression of the same rat Neu cDNA expressed in MMTV-Neu tumours and tumourspheres. Human fibrosarcoma HT1080 cells (Rasheed *et al.*, 1974) were chosen as a parental cell line for their susceptibility to lentiviral infection (Ramezani *et al.*, 2000). HT1080 cells were infected with unconcentrated supernatant containing lenti-Neu, a recombinant self-inactivating lentivirus expressing the rat Neu cDNA. Transduced cells (designated HTN) were

selected in blasticidin-containing media and expanded as a population to verify the expression of Neu by protein analyses (Figure 13). Lysates from wild-type HT1080 and HTN cells, and from 293FT cells transiently transfected with Neu expression plasmid, were compared by Western immunoblot with antibodies to erbB2/Neu (Ab-3, clone 3B5) and loading control β-actin. The low endogenous expression of Neu protein in HT1080 cells (far right lane) suggested this parental cell line was suitable for knockdown studies.

To calculate changes in Neu protein levels from HTN cells by quantitative western blot, we first investigated antibodies Ab-3 (Neu, Oncogene Science) and β-actin loading control (Abcam) in a titration experiment using dilution series of Neu-containing cellular lysate (Figure 14). ImageQuant software calculated the volume of triplicate bands, and their average was plotted as a function of micrograms of loaded protein. Linear regression analyses determined that over a 10-fold range of concentrations, the Neu antibody Ab-3 could reliably detect changes in Neu expression (R-squared value of 0.952) while the β-actin antibody performed with an R-squared value of 0.939 (Figure 14B). These data suggested the suitability of quantitative immunoblot for the quantification of Neu knockdown in adherent HTN cells.

Preparation of recombinant shRNA lentiviruses

The pPRIME lentiviral vector system developed for use in mammalian shRNA screens (Stegmeier *et al.*, 2005) was chosen here for its diversity of optional features including tetracycline-inducible shRNA expression, fluorescent marker GFP, cell surface

marker NGFR, and neomycin selection. These vectors also feature the microRNA context miR30 for the increased processing of shRNAs by cellular RNAi machinery (Paddison *et al.*, 2004). The pPRIME-CMV-GFP vector expresses the GFP-miR30-shRNA fusion message (Stegmeier *et al.*, 2005) from a single PolII promoter (CMV) and was employed to generate lenti-shRNA vectors targeting rat Neu. A total of 12 shRNA sequences (including one scrambled non-silencing control sequence, NSC) were cloned into the pPRIME-CMV-GFP vector; three of these shRNA sequences specific to the rat transgene were chosen for analyses in HTN cells based on pilot shRNA/Neu cotransfection experiments. Constructs R1, R2, R3, and NSC were then used to generate lentiviruses by cotransfection with packaging plasmids in 293FT cells, as described in Materials and Methods.

The titer of concentrated lentiviruses was calculated as described (Rubinson *et al.*, 2003), by infecting wild-type HTN cells with serial dilutions of lentivirus and assaying the fraction of eGFP-positive cells 48 hours post-infection by flow cytometry on the Agilent Bioanalyzer. The titer is reported in Figure 15 as IU/ml or infective units per microliter. In these experiments, the vector pLL3.7b (LentiLox) (Rubinson *et al.*, 2003) was used as a positive control. The mean GFP fluorescence from infected cells in each population was calculated and compared to that of cells infected with LentiLox virus. Confirming our overall observation from fluorescence microscopy, LentiLox infection (Figure 15E) resulted in approximately twofold higher average expression of eGFP per cell when compared with pPRIME vectors in Panels A-D (note the log scale of both axes), suggesting

differences in the efficiency of eGFP transcription or mRNA stability in pPRIME vectors compared to the LentiLox control. Furthermore, when prepared in parallel with pPRIME vectors, the titer of LentiLox virus was more than twofold higher (Figure 15, bottom panel) than the highest titer obtained with a pPRIME vector (non-silencing control, NSC).

Silencing of Neu protein expression in HTN cells by lenti-shRNA infection

HTN cell populations were infected with shRNA lentivirus vectors targeting rat Neu (R1, R2, R3), or a non-silencing control virus (NSC). Expression of GFP was visible in these cells by 24 hours post-infection and robust signal was present an additional 24 hours later. Cell populations infected with various lenti-shRNAs, and cells not infected with any lenti-shRNA (mock-infected) were expanded in culture for 8 days post-infection, with passage at sub-confluence to a larger culture vessel. As the pPRIME-CMV-GFP vector carries no mammalian selectable marker, expression of eGFP was verified qualitatively by fluorescence microscopy just prior to harvest of cell lysates for western blotting to Neu, β-actin and eGFP.

Figures 16 and 17 show the results of two independent biological experiments carried out in technical duplicate for infection of HTN cells with lentiviruses encoding anti-Neu shRNA R1 or non-silencing control (NSC). To demonstrate that increasing doses of anti-Neu shRNA could reduce Neu protein expression, cells were incubated with two quantities of each lenti-shRNA vector (R1 and NSC). Relative Neu protein levels (normalized to β-actin expression from the same sample) were reduced by 85.5% and

84.9% in the first and second experiment, respectively (Figure 16B, 17B). The relative expression of Neu in mock-infected cells was set at 100% in these experiments. Since the pPRIME-CMV-GFP shRNA delivery vector expresses eGFP, western analyses to eGFP protein reveal that increasing doses of lenti-shRNA treatments are responsible for decreases in Neu protein levels (Figure 16A, 17A, bottom immunoblot panel).

Similarly, Figures 18 and 19 (two experiments with lenti-shRNA R2) and Figures 20 and 21 (lenti-shRNA R3) show that the highest dose of lenti-shRNA induced decreases in Neu expression of 63.5% and 63.2% (R2), and 67.5% and 62.3% (R3), respectively compared to expression in HTN cells infected with a corresponding 10 μl dose of non-silencing control virus.

To examine the distribution of eGFP and the corresponding levels of Neu protein in cells infected with lenti-shRNAs, replicate cultures from the above experiments were seeded for analyses by Neu immunocytochemistry 8 days post-infection. Figure 22 shows cells infected with the highest dose (10 μl) of R1, R2, R3, and non-silencing control (NSC) lentivirus vectors. The fraction of cells expressing high or low quantities of Neu protein was counted from multiple fields chosen at random and reported Figure 22R. HTN cells infected with R1, R2, R3, and NSC vectors comprised 6.5%, 10.5%, 11.0%, and 36.7% of cells with high Neu expression, while mock-infected cells contained 38.2% (Figure 22). Similarly, R1, R2, R3, and NSC-infected cells comprised 20.6%, 52.1%, 59.7%, and 60.84% of cells with low Neu expression, while mock infected cells contained 60.5%. We did not detect expression of rat Neu in parental HT1080 cells by

immunocytochemistry (Figure 22PQ). These data suggested that lenti-shRNA infections targeting Neu mRNA reduced both the number of Neu-expressing cells in the HTN population and the level of Neu expression per cell, as scored by immunocytochemistry.

Taken together, our data revealed a set of validated RNAi tools for functional studies in Her2/Neu-induced tumourspheres. We anticipate these experiments will form the baseline in lenti-shRNA screens performed with tumourspheres in the near future.

DISCUSSION

A gene signature for candidate cancer stem cells: *tumourness* genes

Using genes preferential to tumourspheres compared to differentiated mammospheres as a starting point, we have attempted to model the tumour phenotype (Figure 10A). Our data revealed a set of genes whose expression was not appreciably regulated during the differentiation of mammospheres *in vitro*, contrasting with strong regulation in tumourspheres. To eliminate potential *in vitro* artifacts, we have filtered this list of genes to include only transcripts which also showed preferential expression in primary tumours compared with the normal mammary gland. The result is a gene signature, profiled in Figure 10C and listed in Table S5, with high expression in tumour compartments compared to differentiated cells, mature tissue, or proliferating progenitor cells (mammospheres). Among these genes are tight junction proteins of the claudin family and the iron transporter lipocalin 2, whose expression we have verified in primary tumours and tumourspheres by immunohistochemistry (Figure 11). These genes have

characterized roles in Her2/Neu-mediated oncogenesis (Kubben *et al.*, 2007; Morin, 2005; Morohashi *et al.*, 2007), increasing our confidence that tumourness genes are a biologically relevant gene signature. We propose to investigate roles for these genes as biomarkers or therapeutic targets in tumourspheres, a flexible and powerful *in vitro* system.

Use of tumourspheres to screen for novel therapeutic targets

Multiple investigators have reported that maintained Neu expression is required for cell viability in Her2/Neu-overexpressing tumours, a cornerstone of the clinical success enjoyed by Herceptin, an antibody treatment for breast cancer targeting the ectodomain of the Her2/Neu receptor tyrosine kinase. The antibody binds Her2/Neu on the cell surface inducing its internalization and thus ablating its participation in MAP-kinase signal transduction. Similar phenomena are reported in human breast cancer cell lines, where siRNA-mediated knockdown of Her2/Neu causes apoptosis (Choudhury *et al.*, 2004; Faltus *et al.*, 2004), and in mouse models of conditional neu activation, where deinduction of Neu expression results in tumour regression (Moody *et al.*, 2002). Our proof-of-principle experiment is designed to evaluate the potential use of tumourspheres in larger screens, and is based on the high expression and activation of Neu protein in tumourspheres (Figure 12). To successfully compromise Neu expression in tumourspheres *in vitro* and assess the resulting phenotype, we proceeded to design and validate shRNAs targeting rat Neu in an independent cell line, HT1080.

Our experience with HT1080 cells indicated their high performance in lentiviral infections, making them suitable to test our lenti-shRNA vectors prior to use in tumourspheres. We used a lentivirus encoding rat Neu to transduce HT1080 cells, selected the cells using blasticidin and confirmed the expression of Neu thereafter by immunoblotting and immunocytochemistry (Figure 13); we designated the cells HTN. Western analyses with HTN lysates confirmed that antibodies to Neu and β-actin could be used to measure relative quantities of Neu expression following lenti-shRNA treatment (Figure 13).

We next designed a set of short hairpin sequences from the rat Neu coding sequence using the Cold Spring Harbor miR30 program and cloned these oligonucleotides into pPRIME-CMV-GFP. Our experiments to titer these viruses indicated twofold relative lower levels of GFP expression in pPRIME-infected cells compared with a control lentivirus pLL3.7b, and a further twofold lower titer under the best circumstances (Figure 15). Reasons for the observed lower titer in pPRIME vectors remain unknown; however, comparing the plasmid maps of these two vectors reveals the presence of a U6 promoter directly upstream of the CMV-GFP cassette in pLL3.7b that is not present in the pPRIME vectors. While the observed difference in GFP expression could be due to a number of factors, it seems likely that the U6 promoter contributes to increased transcription efficiency. More rigorous investigation may help clarify these observations.

Testing the efficacy of three shRNA lentiviruses compared to a non-silencing control virus containing a scrambled shRNA, we investigated Neu protein levels following infection of HTN cells with these viruses. We were able to demonstrate that increasing doses of shRNAs R1, R2, and R3 led to decreases in protein expression, as quantified in Figures 16-21, while corresponding increases in non-silencing control did not; construct R1 resulted in the highest degree of knockdown (~85%) compared to non-silencing control. GFP expression was monitored in these experiments, confirming that non-silencing control virus exhibits higher titer than the three shRNA constructs. Interestingly, single copies of shRNA seemed unable to overcome Neu expression in HTN cells. Experiments with cloned cell populations containing either single or multiple copies of integrated Neu are required to confirm these kinetics. However, it remains possible that single copies of shRNAs described here will cause knockdown in Her2/Neu-induced tumourspheres sufficient to measure a loss-of-function phenotype *in vitro*.

Subsequent experiments with short hairpins R1 and NSC in tumourspheres should assess sphere formation and apoptosis *in vitro*, and tumourigenic capacity *in vivo* following orthotopic or subcutaneous engraftment. Combined with the use of the tumourness gene signature presented here, the successful completion of these experiments will establish tumourspheres as a useful model for the *in vitro* screening of candidate therapeutic targets using functional genomics.

MATERIALS AND METHODS

Computational analyses

RNA was previously isolated from mammospheres, differentiated mammospheres, tumourspheres, and primary mouse mammary and tumour tissue (Personal communication: Kurpios and Hassell, 2006; Personal communication: Kurpios and Hassell, 2007). Target preparation and microarray hybridization was performed at the Ottawa Genome Centre according to Affymetrix instructions. Raw data from Affymetrix analyses were imported into GeneSpring using default normalization parameters. Student's t-tests and fold change analyses were used to identify genes whose expression differed on a statistically significant basis (95% confidence) by a factor of two. Consistent present calls were required in the numerator. Raw DAT files pertaining to human mammospheres (Dontu *et al.*, 2003a) were reanalyzed according to the above protocol. The NetAffx Analysis Center (Affymetrix) was used to identify probe sets in the MOE430 genome, which correspond to probe sets from the lists of stemness genes previously published (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002). Batch query results were imported into GeneSpring for comparative analyses using the gene list editor. GeneSpring ontologies and the AmiGO browser (Gene Ontology Consortium) were used to identify probe sets associated with various GO biological processes with a maximum overlap p-value of 0.01. Where external analyses of published gene lists were performed, such genes were entered into GeneSpring using the gene list editor for direct comparisons.

Generation of clonable shRNA sequences

The rat Neu mRNA sequence (accession number NM_017003) was uploaded into the Cold Spring Harbor Labs RNAi portal (Paddison *et al.*, 2004; Stegmeier *et al.*, 2005) to generate short hairpin oligonucleotide sequences targeting Rat Her2/Neu mRNA. Desalted sense strand nucleotides with no 5′ or 3′ modifications were ordered from Invitrogen. Oligonucleotides were reconstituted with ddH_2O to 50μM and stored at -20°C. To append restriction endonuclease recognition sites and miR30 microRNA flanking sequences to both ends of the hairpin oligonucleotides, a universal PCR oligonucleotide primer set was ordered from Invitrogen comprising a forward primer (pSM2CF) with sequence 5'-GATGGCTG-CTCGAG-AAGGTATAT-TGCTGTTGACAGTGAGCG-3' and a reverse primer (pSM2R) with sequence 5'-GTCTAGAG-GAATTC-CGAGGCAGTAGGCA-3'. PCR was carried out in a 100μL reaction with the program and reagent components as described previously (Paddison *et al.*, 2004; Stegmeier *et al.*, 2005). Dimethyl sulfoxide (DMSO) and betaine (both Sigma), are included in the PCR mastermix at a final concentration of 5% each to discourage the formation of DNA secondary structure during template amplification. Cleanup of shRNA template PCR was performed by adding an equal volume (100μL) of Phenol:Chloroform:Isoamyl Alcohol (Invitrogen), vortexing briefly but thoroughly, and spinning at 14,000 RPM for 5 minutes at 4°C in a microcentrifuge. The top phase was transferred to a new tube containing 100μL chloroform (ACP), mixed briefly by vortexing and centrifuged at 14,000 RPM for 5 minutes at 4°C. The top phase was again

transferred to a new tube with 1μL molecular biology grade glycogen and 1:10 volume of 3.0M NaAc, pH 5.2 (sodium acetate). To precipitate the DNA, three volumes of 100% ethanol were added to each preparation and precipitates were pelleted at 14,000 RPM for 15 minutes at 4°C. Pellets were washed in 300μL of 70% ethanol, re-pelleted at top speed for 5 minutes at 4°C, and resuspended in 41.5μL ddH_2O for restriction digest. PCR products and lentiviral vectors were digested for 3-5 hours, separated by agarose electrophoresis on 2% and 0.8% gels, respectively, and purified using MinElute PCR and QIAquick gel extraction columns, respectively (Qiagen). Concentration of eluted products were estimated using an Invitrogen low mass or high mass DNA ladder and ligation reactions were set up for each hairpin construct at a molar ratio of 3:1 (ends of insert : ends of vector). Ligations of 10μL were carried out at 16°c overnight and 5μL were transformed into DH5α subcloning efficiency cells.

Production of Recombinant Lentivirus

Lentiviruses were packaged by transient transfection of 293FT cells with a 2:1:1:1 molar ratio of lentiviral expression vector (pPRIME, pLL3.7b or pLenti6/V5-Neu) and third generation Invitrogen packaging plasmids pLP1 (Gag/Pol), pLP2 (Rev), and pLP-VSV/G (envelope), using Lipofectamine2000 reagent (Invitrogen). 12 x 10^6 293FT cells were seeded 24 hours prior to transfection in 150mm plates. Media was replaced just prior to transfection with media lacking antibiotics, and DNA/Lipofectamine2000 complexes were added according to the manufacturer's instructions. The following morning, the

transfection mix was aspirated and replaced with fresh media containing antibiotics and cells were incubated for a further 24-48 hours prior to harvest of viral supernatant. At harvest, culture supernatants were pooled (in general six plates per construct, 20ml per plate), cleared by centrifugation at 1500g for 15 minutes at 4°C, filtered through ultra-low protein binding PVDF units (0.45μm, Millipore), and split into 40ml fractions. Lenti-Neu virus was stored at -80°C without concentration. Lenti-shRNA vectors were concentrated by ultracentrifugation with a Beckman-Coulter SW32ti Rotor at 28,000 rpm for 90 minutes at 4°C and the pellets were resuspended in 100-600 μL cold PBS supplemented with 1% BSA. Lenti shRNA stocks were then titered essentially as described (Rubinson *et al.*, 2003) by infection of HT-1080 cells with serial dilutions of virus in the presence of 6μg/ml polybrene. Forty-eight hours post-infection, eGFP-positive cells were quantified by cell assay on the Agilent Bioanalyzer using wells which contained approximately 1-10% eGFP positive cells. The titer was then calculated: *number of cells plated* x *fraction of eGFP positive cells* x *dilution factor,* reported as infective units per micoliter (IU/μL). These infections were carried out in duplicate and averaged.

Derivation of Neu-expressing stable cell line HTN

Parental HT-1080 cells were seeded at a density of 1 x 10^5 per 6-well multidish 24 hours prior to infection. The following day, the culture media was replaced with 1 mL of unconcentrated Lenti-Neu culture supernatant for a period of 12 hours, followed by a media change. At 24 hours post-infection, three replicate infections (and two uninfected

negative control wells) were reseeded in 100 mm dishes and subject to selection in blasticidin-containing media to generate HTN cell populations. Cell death was observed at the first media change (4 days) in negative control wells while very few dead cells were observed in transduced HTN cultures. At 8 days post-infection, no viable cells remained in negative control cultures compared to subconfluent transduced cultures, which were reseeded for a further 4 days of selection. Independent HTN populations were maintained thereafter with periodic selection in media containing blasticidin.

Immunocyctochemistry and Immunohistochemistry

Paraffin sections (5-7 μm) of primary tumours were analyzed by immunohistochemistry as described previously (Pechoux *et al.*, 1999) using the antibodies and washing regimen described below. Cells were affixed to coated Cytospin slides (ThermoShandon) or prepared by seeding at appropriate densities in 4-well chamber slides (Nunc) or 24-well multidishes (Corning) 24 hours prior to analysis by immunocytochemistry. Slides/wells were washed 3 times in cold PBS with 0.03% Tween-20 (PBS-T.03), fixed for 20 minutes in 4% paraformaldehyde at room temperature (RT) and washed again 3 times (3x) with PBS-T.03. Blocking was carried out with 5% normal goat serum (NGS) (Dako) in PBS-T.03 for 45 minutes at RT. Primary antibodies were added at in 5% NGS/PBS-T.03 for 1 hour at RT, followed by 3x 5 minute washes with PBS-T.03. Secondary anti-mouse IgG (AlexaFluor 594 signal amplification kit) was added at 1:1000 in 5% NGS/PBS-T.03 for 25 minutes per component with 3x 5 minute washes with PBS-T.03 between and after

secondary antibody incubations. Cells were coverslipped with ProLong Gold mounting media with DAPI (Invitrogen) and analyzed by fluorescence microscopy using a Leica inverted microscope and OpenLab software. The proportion of positive cells and DAPI-stained nuclei was quantified from multiple fields chosen at random, sufficient to count 100 cells or more. Primary monoclonal antibodies: mouse anti-rat Neu (Ab-9, Labvision/NeoMarkers, 1:100); rat anti-mouse TenascinC (R&DSystems). Primary polyclonal antibodies: rabbit anti-mouse PEA3 (PC2, 1:200); rabbit anti-mouse Claudin3, Claudin7 (Abcam), and rabbit anti-mouse Lipocalin2 (culture supernatant, a generous gift from Dr. Nilsen-Hamilton, Iowa).

qRT-PCR

Total cDNA prepared previously (Barcelon, 2006; Personal communication: Kurpios and Hassell, 2006) from passage 2 mammospheres and mammospheres induced to differentiate was subjected to 40 rounds of PCR amplification on the Roche LightCycler using primers to mouse PEA3 and β2-microglobulin (internal control) and SybrGreen DNAMasterI PCR mastermix, according to the manufacturer's instructions. Expression levels of PEA3, ERM, and ER81 were quantified using the standard curve method, normalized to the corresponding levels of the internal control, and were verified with melting curve analysis and DNA electrophoresis for product specificity.

Western Immunoblotting

Monolayers of cells were washed twice with ice cold PBS, and pelleted by centrifugation at 5000rpm in 1ml cold PBS. Cells were lysed in modified RIPA lysis buffer (1% TritonX-100, 0.1% SDS, 1% sodium deoxycholate, 150mM NaCl, 2mM EDTA, 50mM NaF, 1mM sodium orthovanadate, with Complete Mini protease inhibitor cocktail (Roche)) on ice for 30 minutes and quantified using Bradford assay (BioRad). Protein was separated in 8% SDS-PAGE and transferred to PVDF membrane (Immobilon-P, Millipore) in a BioRad TransBlot apparatus. Membrane strips were blocked for 1 hour at RT in a 10% solution of skim milk powder in PBS-Tween20 0.1% (PBS-T.1). Primary anti-neu (Ab-3, Oncogene Science), anti-β-actin antibody (Abcam), or anti-GFP (Clontech) was added in a 10% solution of skim milk powder (in PBS-T.1) for 2 hours at room temperature and subsequently washed with three changes of PBS-T.1 for 5 minutes each. Secondary goat anti-mouse HRP-conjugated antibody (Sigma) was added in 2%/1% blocking solution for 45 minutes at room temperature and again washed 3 x 5 minutes in PBS-T.1. Staining was detected with ECL-Advance chemiluminesence and exposed to ECL film (GE Healthcare) or scanned with a Typhoon TRIO. Duplicate or triplicate bands were detected in ImageQuant software using lanes of equal dimensions with the rubber band method of background subtraction. For each experiment, we report the average relative expression of Neu (vs β-actin) and set mock-infected samples set at 100%.

Cell culture

Mammospheres and tumourspheres were isolated and cultured in serum-free stem cell media as described (Kurpios, 2005). HT1080 and 293FT cells were maintained in high glucose DMEM with 10% FBS, L-Glutamine, 1x Sodium pyruvate, 1x MEM-non-essential amino acids, Pen/Strep and Fungizone (all Invitrogen).

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APPENDIX 1

FIGURES AND TABLES

Table 1. Ontological analyses of genes preferential to mammospheres and mammospheres induced to differentiate

Mammospheres

GO Biological Process		p-value	overlap
cell cycle		**1.75E-21**	**59 / 329**
Ccna2	cyclin A2		
Ccnb2	cyclin B2		
Ccnd1	cyclin D1		
Cdk2	cyclin-dependent kinase 2 isoform 2		
Cdk4	cyclin-dependent kinase 4		
Cks1b	CDC28 protein kinase 1b		
cell proliferation		**0.000328**	**21 / 217**
	positive regulation of cell proliferation	0.000755	7 / 37
Cdk4	cyclin-dependent kinase 4		
c-kit	truncated tyrosine kinase receptor; c-kit		
Ereg	epiregulin		
Ki-67	Mki-67		
S100a6	S100 calcium binding protein A6 (calcyclin)		
Tgfb1	transforming growth factor, beta 1		
DNA replication		**1.44E-09**	**21 / 103**
	DNA replication initiation	1.45E-07	7 / 12
Pcna	proliferating cell nuclear antigen		
Pola1	polymerase (DNA directed), alpha 1		
Pole2	DNA polymerase epsilon small subunit; DNA polymerase epsilon subunit 2		
Prim1	primase p49 subunit; DNA primase small subunit, 49kDa		
Rpa2	replication protein A2		
DNA packaging		**0.000221**	**16 / 138**
H2afx	H2A histone family, member X		
H2afz	H2A histone family, member Z		
Hat1	histone aminotransferase 1		
Hells	helicase, lymphoid specific		
Smarca5	ATP-dependent chromatin remodeling protein SNF2H; SWI/SNF related, matrix associated, actin		
DNA metabolism		**3.06E-13**	**53 / 410**
	DNA modification	3.93E-05	8 / 32
	DNA methylation	1.79E-05	8 / 29
Dnmt1	DNA methyltransferase (cytosine-5) 1		
Hmga1	high mobility group AT-hook 1 isoform I		
Hmga2	high mobility group AT-hook 2		
Hmgb3	high mobility group box 3		
Rad21	RAD21 homolog		
microtubule-based process		**8.28E-05**	**19 / 168**
Kif18a	kinesin family member 18A		
Nusap1	nucleolar protein ANKT		
RanBP1	Ran/TC4 Binding Protein		
Stmn1	stathmin 1		
Tubb6	tubulin, beta 6		
mRNA processing		**0.00099**	**20 / 220**
Cpeb1	cytoplasmic polyadenylation element-binding protein		
Hnrpa2b1	heterogeneous nuclear ribonucleoprotein A2/B1 isoform 1;		
Lsm5	LSM5 homolog, U6 small nuclear RNA associated		
Refbp2	RNA and export factor binding protein 2		
Sf3a3	splicing factor 3a, subunit 3		
Sfpq	PTB-associated splicing factor		
cell adhesion		**0.00041**	**37 / 489**
	focal adhesion formation	0.00993	2 / 4
Cd34	CD34 antigen		
Cd44	CD44		
Col5a3	procollagen, type V, alpha 3		
Icam1	intercellular adhesion molecule		
Itgav	integrin alpha V		
Lama2	laminin-2 alpha2 chain precursor		
lipid metabolism		**0.00343**	**29 / 403**
	fatty acid elongation	0.00511	2 / 3
Elovl6	ELOVL family member 6, elongation of long chain fatty acids		
Hsd17b12	hydroxysteroid (17-beta) dehydrogenase 12 protein		
Lipg	lipase, endothelial		
Mgll	monoglyceride lipase		
Pla2g7	phospholipase A2, group VII		
Rbp1	retinol binding protein 1, cellular		
negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism		**0.00509**	**8 / 64**
Atpif1	ATPase inhibitory factor 1		
Bhlhb2	basic helix-loop-helix domain containing, class B2		
Psmc5	protease (prosome, macropain) 26S subunit, ATPase 5		
Tbx2	T-box 2		
Th1l	TH1 homolog		
collagen catabolism		**0.00218**	**4 / 14**
Mmp9	matrix metalloproteinase 9		
Mmp10	matrix metalloproteinase 10		
Mmp14	matrix metalloproteinase 14 (membrane-inserted)		
chromosome organization and biogenesis		**8.28E-05**	**19 / 168**
Fen1	flap structure specific endonuclease 1		
Mcm2	minichromosome maintenance deficient 2 mitotin		
Rrm1	ribonucleotide reductase M1		

Mammospheres induced to differentiate

GO Biological Process		p-value	overlap
chemotaxis		**0.000914**	**9 / 108**
	induction of positive chemotaxis	0.0051	2 / 5
Aspn	asporin		
Cxcl12	stromal cell derived factor 1 isoforms alpha, beta, gamma precursor		
Cxcl5	chemokine (C-X-C motif) ligand 5		
Fmod	fibromodulin		
Il1b	interleukin 1, beta		
Slit2	SLIT2; slit homolog 2		
cell adhesion		**1.54E-07**	**32 / 489**
Adam12	a disintegrin and metalloprotease domain 12 (meltrin alpha)		
Col1a1	collagen pro-alpha-1 type I chain; procollagen, type I, alpha 1		
Col5a1	procollagen, type V, alpha 1		
Fbln5	fibulin 5		
Itgbl1	integrin, beta-like 1		
Lama4	laminin, alpha 4		
Ncam1	neural cell adhesion molecule		
signal transduction		**0.000343**	**62 / 1721**
	insulin-like growth factor receptor signaling pathway	1.39E-06	4 / 5
	platelet-derived growth factor receptor signaling pathway	0.00311	2 / 4
	regulation of Notch signaling pathway	0.000534	2 / 2
	Wnt receptor signaling pathway	0.000219	9 / 89
Angpt4	angiopoietin 4		
Ctgf	connective tissue growth factor		
Fst	follistatin		
Fzd2	frizzled 2		
Igf1	insulin-like growth factor 1		
Il1r1	interleukin 1 receptor, type I		
IL1RA	interleukin 1 receptor antagonist		
Il6st	interleukin 6 signal transducer		
Pdgfc	platelet-derived growth factor, C polypeptide		
Sfrp1	secreted frizzled-related sequence protein 1		
Sfrp2	secreted frizzled-related sequence protein 2		
Wisp1	WNT1 inducible signaling pathway protein 1		
Wnt5a	wingless-related MMTV integration site 5A		
cell death		**0.00634**	**18 / 403**
	programmed cell death	0.00665	17 / 374
	negative regulation of apoptosis	0.00885	6 / 77
Atf5	activating transcription factor 5		
Bnip3	BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3		
Dapk1	death associated protein kinase 1		
Ddit3	DNA-damage inducible transcript 3		
Gadd45b	growth arrest and DNA-damage-inducible 45 beta		
Gas2	growth arrest specific 2		
Trib3	tribbles homolog 3		
cellular morphogenesis		**1.13E-07**	**22 / 252**
	regulation of cell size	3.59E-17	19 / 59
	cell growth	7.86E-18	19 / 55
Gas6	growth arrest specific 6		
Igfbp2	insulin-like growth factor binding protein 2		
Nedd9	neural precursor cell expressed, developmentally down-regulated gene 9		
Nov	nephroblastoma overexpressed gene		
Prss11	protease, serine, 11 (Igf binding)		
Socs3	suppressor of cytokine signaling 3		
generation of precursor metabolites and energy		**0.00931**	**19 / 451**
Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1		
Eno1	enolase 1, alpha non-neuron		
Fads3	fatty acid desaturase 3		
Gaa	glucosidase, alpha, acid		
H6pd	hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)		
Pfkl	phosphofructokinase, liver, B-type		
regulation of phosphorylation		**0.0061**	**4 / 32**
	regulation of protein amino acid phosphorylation	0.00425	4 / 29
Pdgfc	platelet-derived growth factor, C polypeptide		
Pdgfd	platelet-derived growth factor D		
Socs3	suppressor of cytokine signaling 3		
organ development		**3.78E-06**	**45 / 941**
	blood vessel morphogenesis	0.00816	7 / 99
	angiogenesis	0.0038	7 / 86
	positive regulation of odontogenesis	0.00158	2 / 3
	skeletal development	0.00519	7 / 91
Acta2	actin, alpha 2, smooth muscle, aorta		
Aes	amino-terminal enhancer of split		
Cryab	crystallin, alpha B		
Ephb3	Eph receptor B3		
Figf	c-fos induced growth factor		
GDNFR-beta	glial cell line derived neurotrophic factor family receptor alpha 1		
Id3	inhibitor of DNA binding 3		
Myh9	myosin, heavy polypeptide 9, non-muscle isoform 1		
Sema3c	semaphorin 3C		
Tgfb3	transforming growth factor, beta 3		
Vegfa	vascular endothelial growth factor A		
bone remodeling		**0.00875**	**5 / 55**
Csf1	colony stimulating factor 1		
Nox4	Nox4 protein; NADPH oxidase 4		

Table 2. List of β-catenin signaling pathway components preferentially expressed in mammospheres (UMS) or mammospheres induced to differentiate (DMS).

Symbol	Gene name	Fold change in		Regulation Activity	Function
		UMS	DMS		
Wnt2	wingless integration site 2	19.83		+	secreted: activates signaling via frizzled binding
Fzdb	frizzled-related protein	10.14		-	secreted: competes with frizzled receptors for wnt binding
Tcf7	T-cell factor 7	3.91		+	co-activates transcription with β-catenin
NLK	nemo-like kinase	3.74		-	inhibitis Tcf activity; activated by TGF-β
Tcf4	T-cell factor 4	3.19		+	co-activates transcription with β-catenin
Pin1	peptidyl-prolyl cis/trans isomerase) NIMA-interacting	2.09		+	stabilizes β-catenin
Tcf19	T-cell factor 19	2.06		+	co-activates transcription with β-catenin
Axin2	Axin-2	2		-	β-catenin destruction complex
Sfrp2	secreted frizzled-related protein 2		27.99	-	secreted: competes with frizzled receptors for wnt binding
Sfrp1	secreted frizzled-related protein 1		14.04	-	secreted: competes with frizzled receptors for wnt binding
Dact1	dapper homolog 1		4.11	-	binds disheveled; inhibits β-catenin signal transduction
Fzd2	frizzled homolog 2		3.59	+	extracellular wnt receptor
Fzd8	frizzled homolog 8		3.36	+	extracellular wnt receptor
Wnt5a	wingless integration site 5a		2.15	+	secreted: activates signaling via frizzled binding; non-canonical

Symbol	Gene name	# of PEA3 sites	Fold change in	
			UMS	DMS
Etv4	ets variant gene 4 (E1A enhancer binding protein, E1AF); PEA3		38.97	
Ptgs2	cox-2; prostaglandin endoperoxide synthase 2		37.24	
Gsta4	glutathione S-transferase, alpha 4		34.17	
Dusp6	dual specificity phosphatase 6		27.02	
Plf	proliferin		18.7	
Cldn1	claudin 1		14.77	
Ccl2	chemokine (C-C motif) ligand 2	9	7.48	
Dlk1	delta-like 1		6.05	
Runx2	runt related transcription factor 2		6.04	
Ccnd1	cyclinD1		5.59	
Idb2	inhibitor of DNA binding 2		5.39	
Sdc4	syndecan 4	3	5.38	
Ereg	epiregulin		4.96	
Kitl	kit ligand		4.24	
Tfrc	transferrin receptor		4.21	
Robo1	roundabout homolog 1 (Drosophila)		3.9	
Ank	progressive ankylosis		3.89	
Tgfbr1	TGF receptor beta -1		3.66	
Ccna2	cyclinA2		3.35	
Gnpi	glucosamine-6-phosphate deaminase 1		3.2	
Lcn2	lipocalin 2	4	3.12	
Egr1	early growth response 1	2	3.02	
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase		2.92	
MMP14	matrix metalloproteinase 14 (membrane-inserted)		2.81	
Pcdh7	proto-cadherin 7		2.7	
Il1rl1	interleukin 1 receptor-like 1		2.62	
Cntn1	contactin 1		2.57	
Spp1/Opn	Osteopontin/Secreted phosphoprotein-1	3	2.49	
Jun	jun oncogene		2.26	
Tyms	thymidylate synthase	5	2.17	
Elk3	ELK3, member of ETS oncogene family		2.15	
Rfc4	replication factor C (activator 1) 4	2	2.1	
CD44	CD44 antigen		2.1	
H2-D1	H2D1 immunoglobulin heavy chain		2.02	
Axin2	Axin-2 (component of Wnt signaling)		2	
Idb4	inhibitor of DNA binding 4			31.65
Cxcl5	chemokine (C-X-C motif) ligand 5	5		30.77
Ctgf	connective tissue growth factor			10.87
Wisp2	WNT1 inducible signaling pathway protein 2	2		9.04
Cyr61	cysteine rich protein 61			8.03
Fzd2	frizzled homolog 2 (component of Wnt signaling)			5.53
Il1rn	interleukin 1 receptor antagonist	3		5.44
col8a1	procollagen, type XVIII, alpha 1	7		4.792
Col1a1	procollagen, type I, alpha 1	4		4.43
Igfbp7	insulin-like growth factor binding protein 7			3.64
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	5		3.43
Lbp	lipopolysaccharide binding protein			3.36
Dusp1	dual specificity phosphatase 1			3.29
Fst	follistatin			2.94
VEGFa	vascular endothelial growth factor a			2.42
Myh9	myosin, heavy polypeptide 9, non-muscle	1		2.34
Wisp1	WNT1 inducible signaling pathway protein 1			2.2
Wnt5a	ingless-related MMTV integration site 5a (component of Wnt signaling)			2.15
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta			2.13
Pedf	pigment epithelium-derived factor	2		2.13
Adam12	a disintegrin and metallopeptidase domain 12 (meltrin alpha)			2.1
Actb	actin beta (cytoplasmic)	2		2.093

Table 3. Known Wnt/β-catenin target genes are differentially expressed in mammospheres and differentiated cells. Many genes known to be regulated by Wnt/β-catenin signaling are differentially expressed in both mammospheres and differentiated cells, suggesting complex regulation of Wnt signaling in mammospheres. Because ETS transcription factor PEA3 is known to cooperate with β-catenin in the activation of transcription, genes with multiple PEA3 binding sites upstream of their transcriptional start site have been indicated.

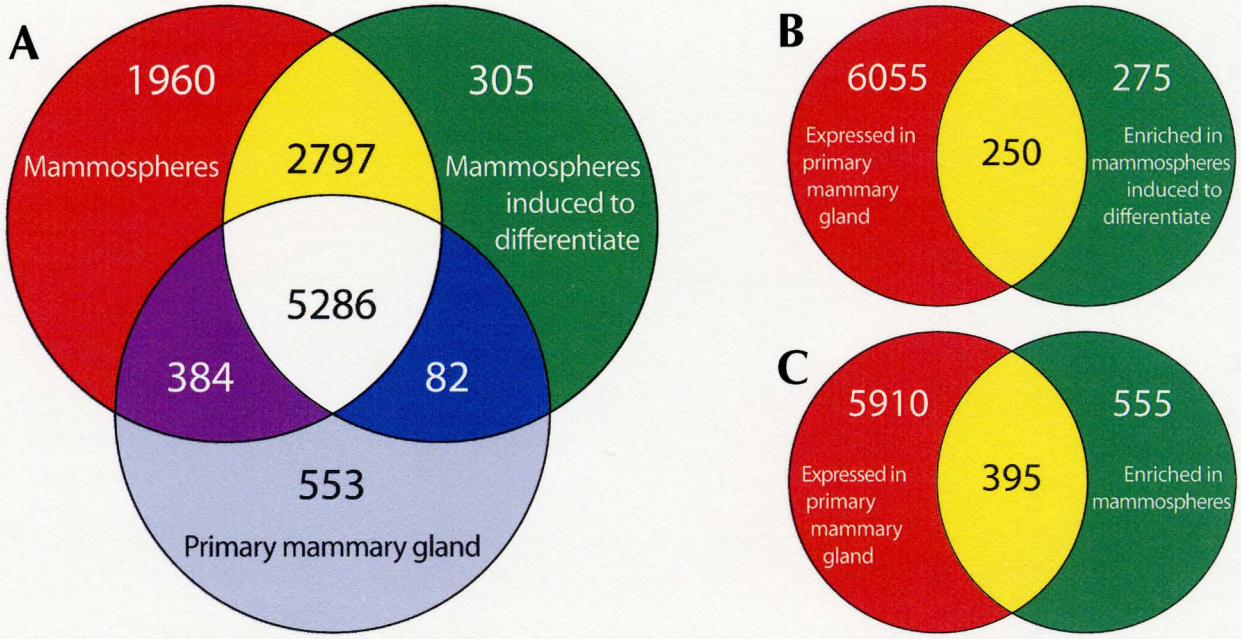


Figure 1 Mammospheres express genes in common with cells of the wild type mammary gland. Genes scored with present calls from two replicate samples deriving from each of 5, 10, and 15-week old virgin FVB mice were pooled (present calls in 6 of 6 samples) to establish a cohort of genes with consistent detectable expression in the mammary gland, independent of preferential expression at any timepoint. **(A)** This list of expressed genes was compared against mammospheres and mammospheres induced to differentiate, revealing that the expression of many genes was maintained over the course of *in vitro* propagation and differentiation. To learn what proportion of genes preferential to either cell population were similarly expressed in primary mammary tissue, lists from mammospheres induced to differentiate *in vitro* **(B)** or mammospheres **(C)** were overlapped with present calls in the mammary gland to quantify the proportion of genes potentially induced as a consequence of culturing the cells *in vitro*.

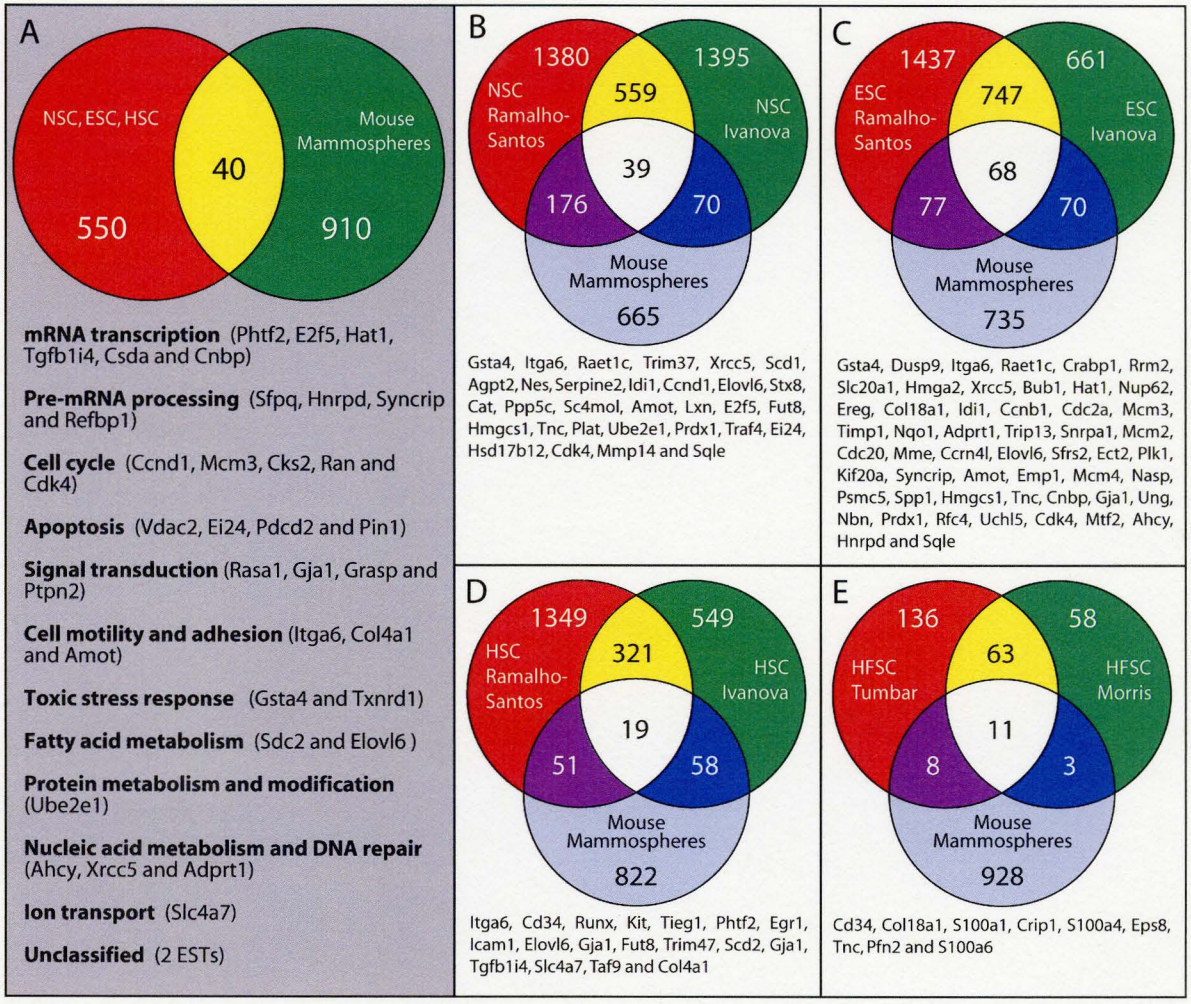


Figure 2 Mammospheres preferentially express genes characteristic of stem cells from multiple tissues. Genes preferential to nerual (NSC), embryonic (ESC), and hematopoeitic stem cells (HSC) were identified from two independent reports of "stemness" transcriptional profiling. **(A)** The combined list of stem cell genes was overlapped with transcripts preferential to mammospheres in culture to reveal 40 genes in common. Transcripts demonstrated in independent reports to be preferential to stem cells from **(B)** neural, **(C)** embryonic, **(D)** hematopoietic, and **(E)** hair follicle stem cells are overlapped with mammosphere-specific genes. Previously established markers of early progenitors or stem cells from various tissue compartments are counted among the genes in common to mammospheres and the indicated stem cell populations.

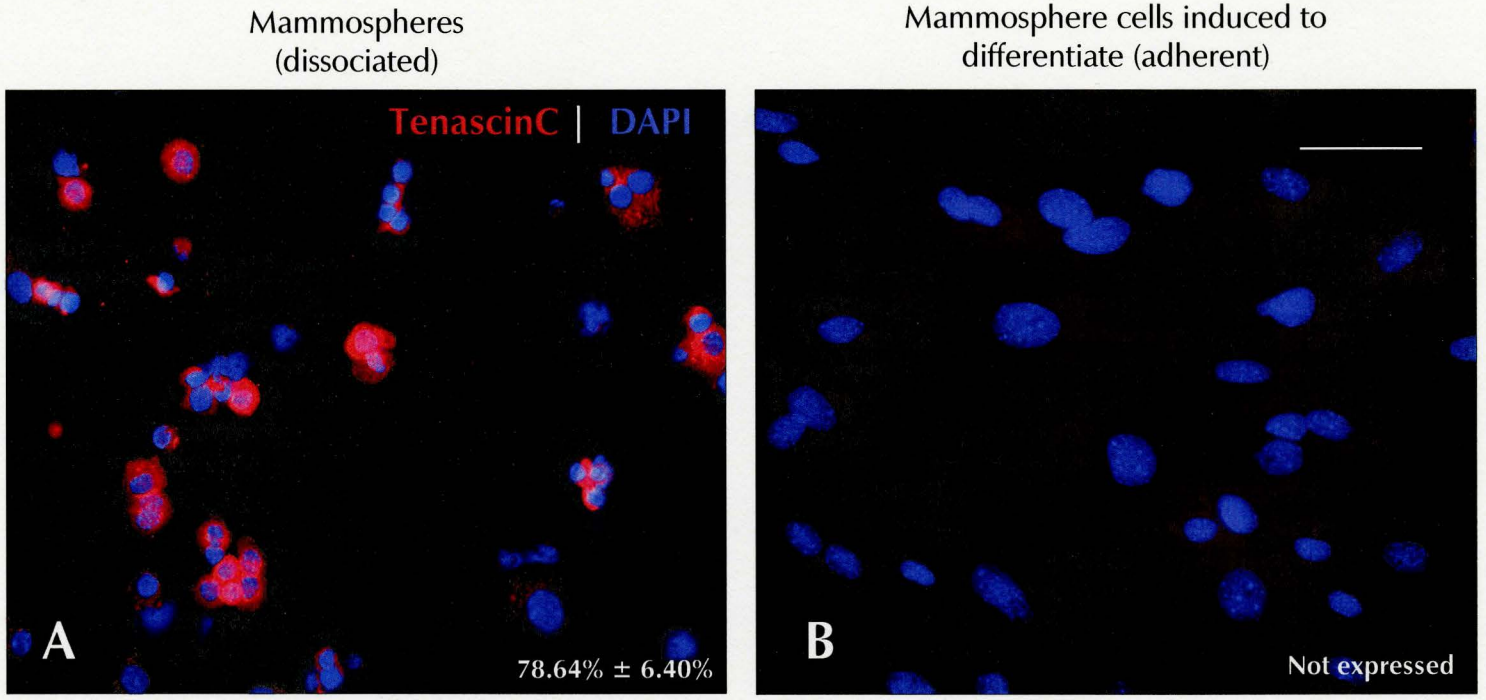


Figure 3. Differential expression of tenascinC in mammospheres and mammospheres induced to differentiate. TenascinC (RED) was identified as preferentially expressed in mammospheres **(A)** compared to differentiated cells **(B)**, and is one of the "stemness" genes expressed among enriched populations of neural, embryonic, and hair follicle stem cells. Thus tenascinC may play a role in maintaining the multpotency of mammosphere-resident cells, or may contribute to their niche. DAPI staining (blue) marks nuclei. Scale bar, 100 μm.

Figure 4. Human and mouse mammospheres preferentially express few genes in common. Transcripts preferential to mammospheres **(A)** and differentiated mammospheres **(B)** were overlapped with corresponding samples from human cells, illustrating a low correspondence of these data considering the similarity of the tissues of origin.

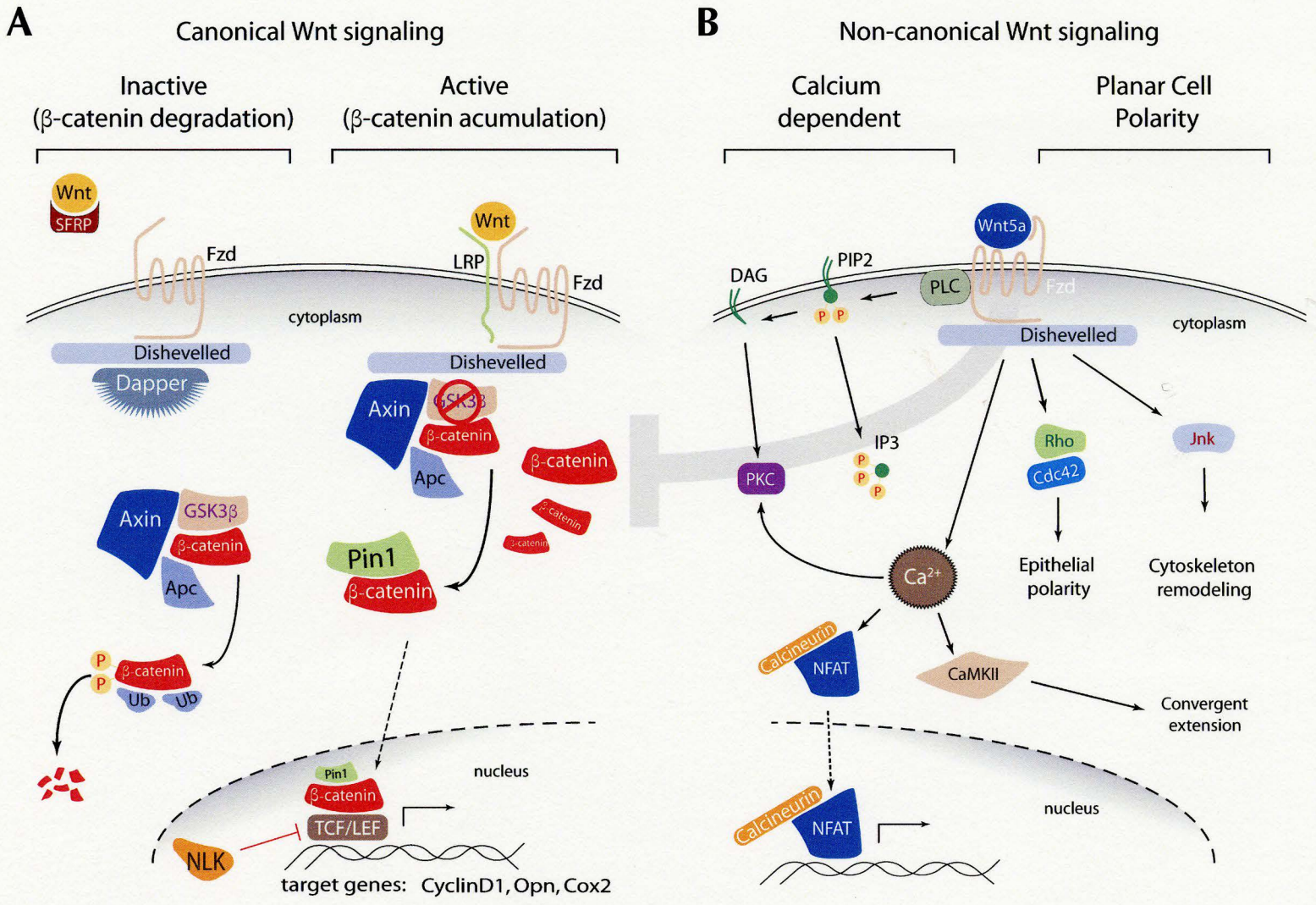


Figure 5. Schematic of canonical and non-canonical Wnt/β-catenin signaling . In the absence of soluble secreted Wnt proteins **(A)**, β-catenin is bound by the scaffolding protein Axin, adenomatous polyposis coli (APC), and glycogen synthase-kinase 3β (GSK3β) which results in its phosphorylation. β-catenin is then ubiquitinated and degraded. **Canonical signaling** is activated when Wnt proteins stimulate membrane-bound frizzled receptors to activate disheveled, which binds GSK3β ultimately permitting accumulation of β-catenin. Free β-catenin translocates to the nucleus to participate in Tcf/Lef-dependent transcription of target genes. **(B) Non-Canonical signaling** of β-catenin involves the activation of frizzled by Wnt5a and induces changes in cytoskeletal organization and cell polarity.

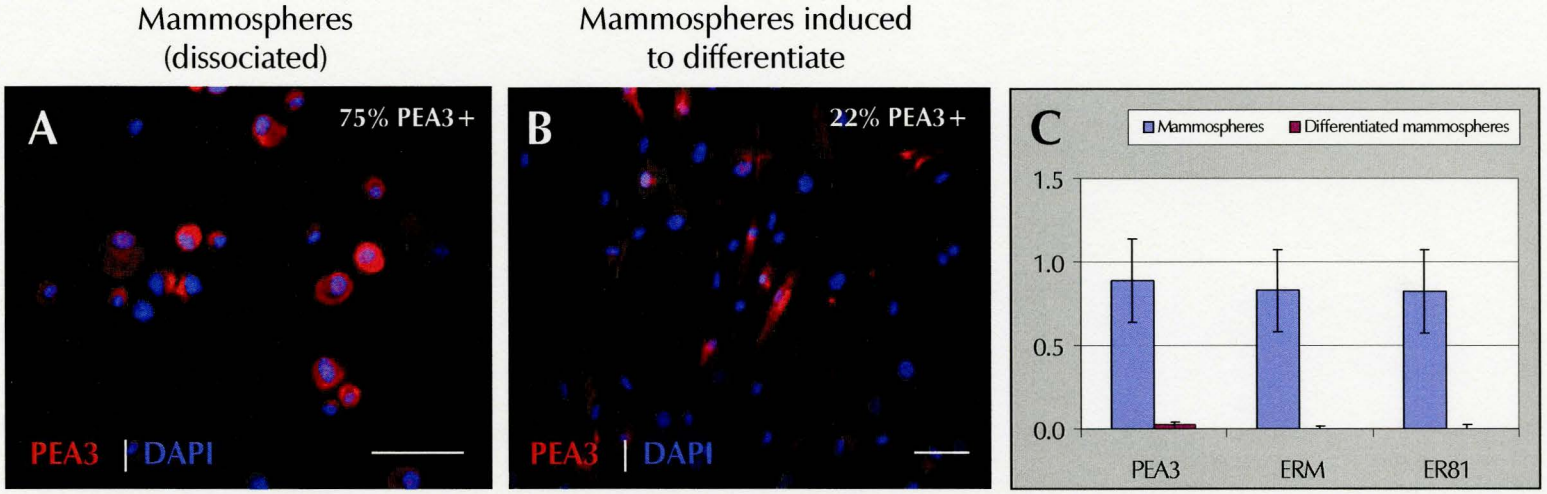


Figure 6. Differential expression of PEA3 and its subfamily members in mammospheres and differentiated mammospheres. (A) PEA3 is expressed in 75% of mammosphere cells, but only in 22% **(B)** of differentiated mammosphere cells. Scale bars, 100 μm. **(C)** Members of the PEA3 subfamily of Ets transcription factors are preferentially expressed in mammospheres compared to differentiated cells, as verified by qRT-PCR. Levels of expression and standard deviations are reported for mammospheres and mammospheres induced to differentiate *in vitro*. Quantitative RT-PCR was performed using the standard curve method, where all concentrations were normalized to the internal control gene β2-microglobulin.

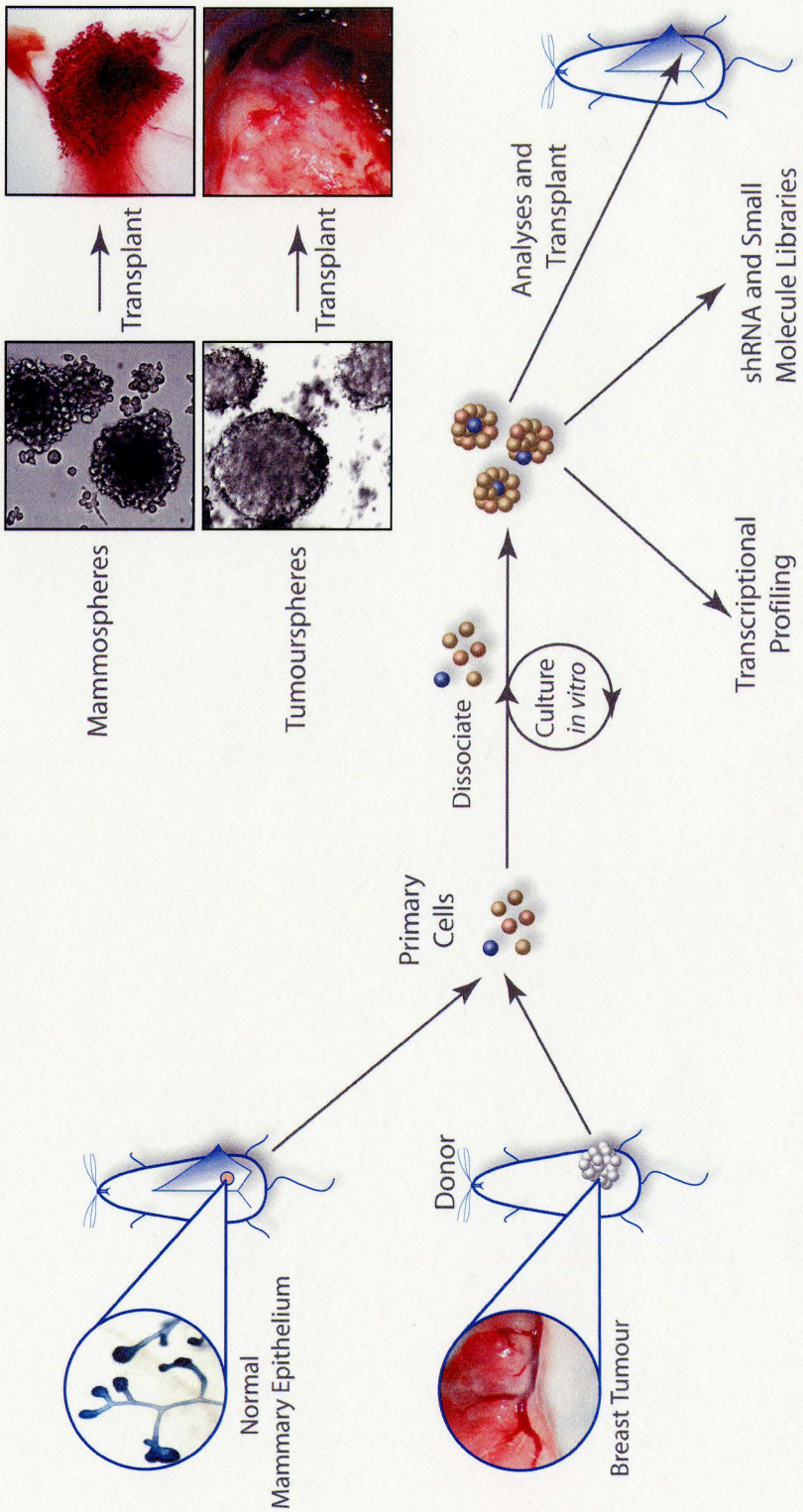


Figure 7. Schematic of tumoursphere culture model.

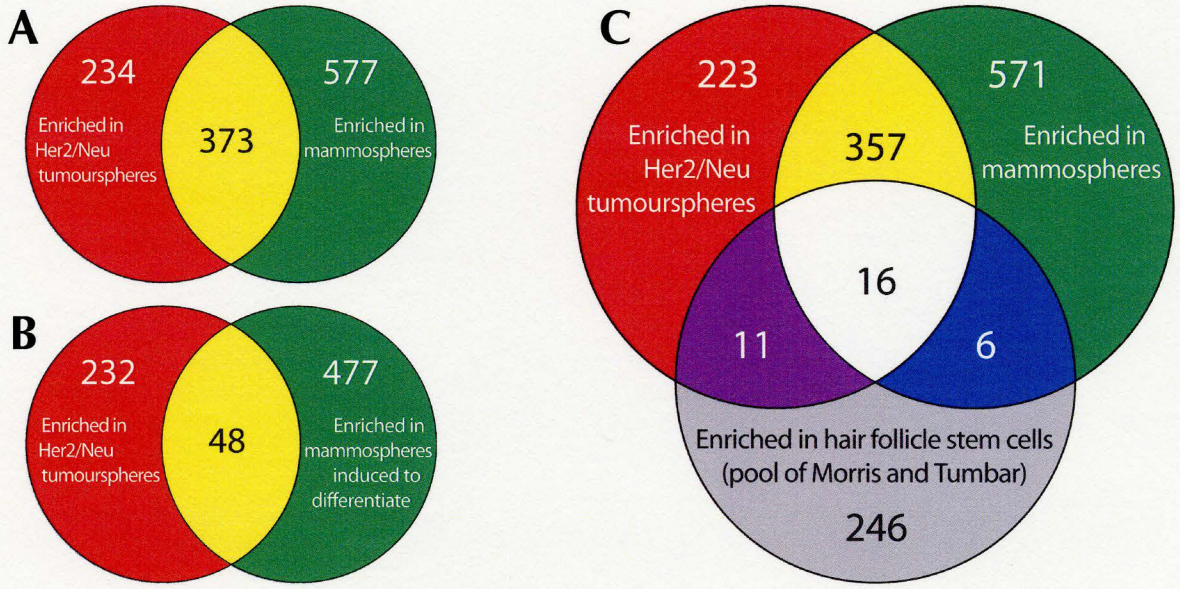


Figure 8. Tumourspheres express an amplified stem cell signature. Genes preferentially expressed (enriched) in tumourspheres overlap more significantly with those enriched in mammospheres **(A)**, than they do with those enriched in differentiated mammospheres **(B)**. Also, tumourspheres exhibit greater overlap with hair follicle stem cells **(C)** than mammospheres do, suggesting an amplified stem cell phenotype in tumourspheres.

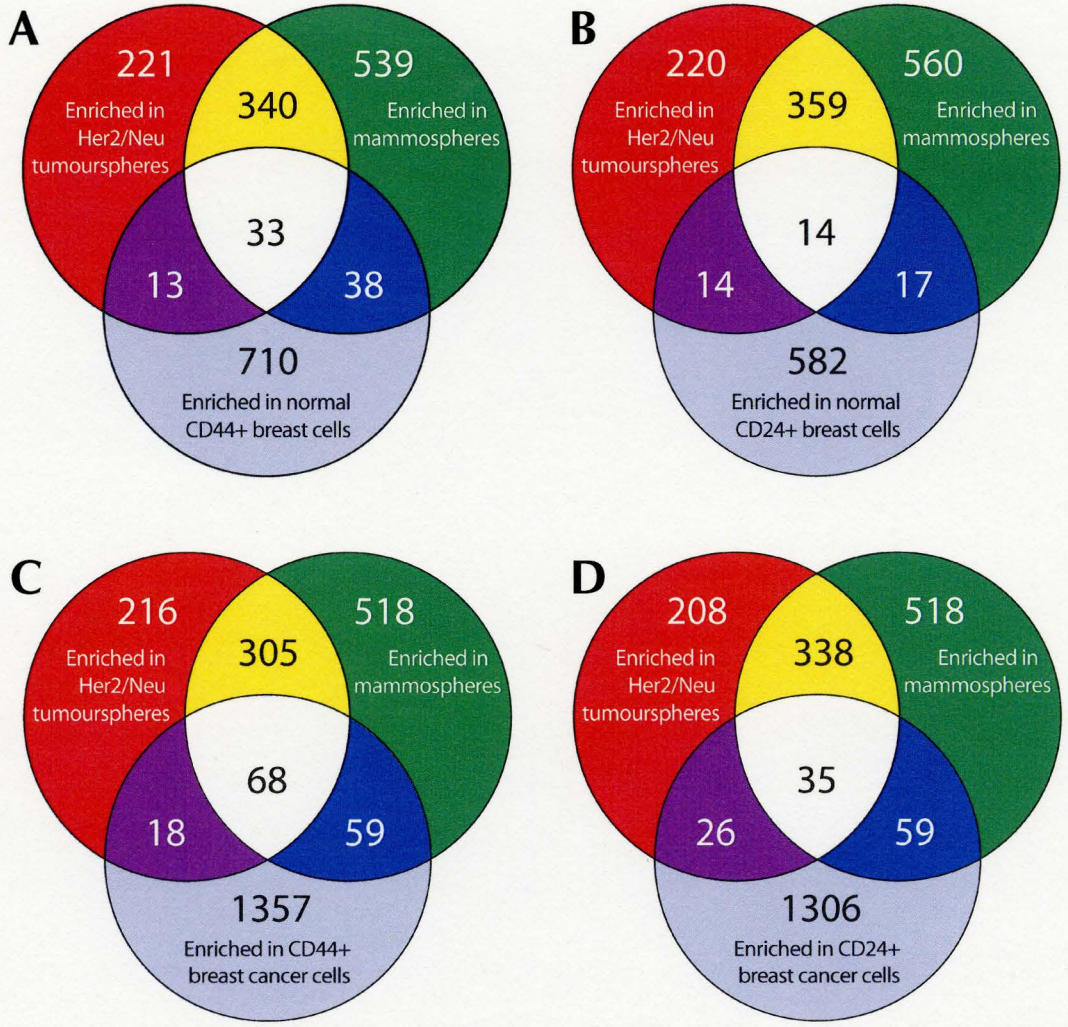


Figure 9. Tumourspheres display similarity to both CD44+ and CD24+ prospective human breast cancer cells. Genes preferentially expressed (enriched) in human CD44+/CD24- (referred to as CD44+) and CD24+/CD44- (CD24+) cells from normal and tumour breast compartments are overlapped with genes preferentially expressed (enriched) in tumourspheres and mammospheres, showing greater overlap of tumourspheres with normal and tumour CD44+ cells .

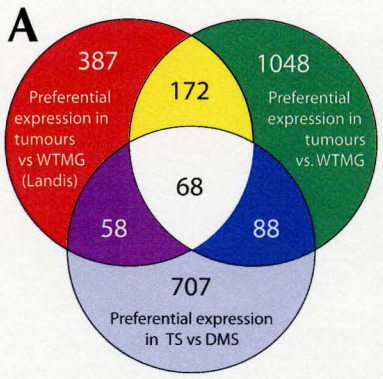
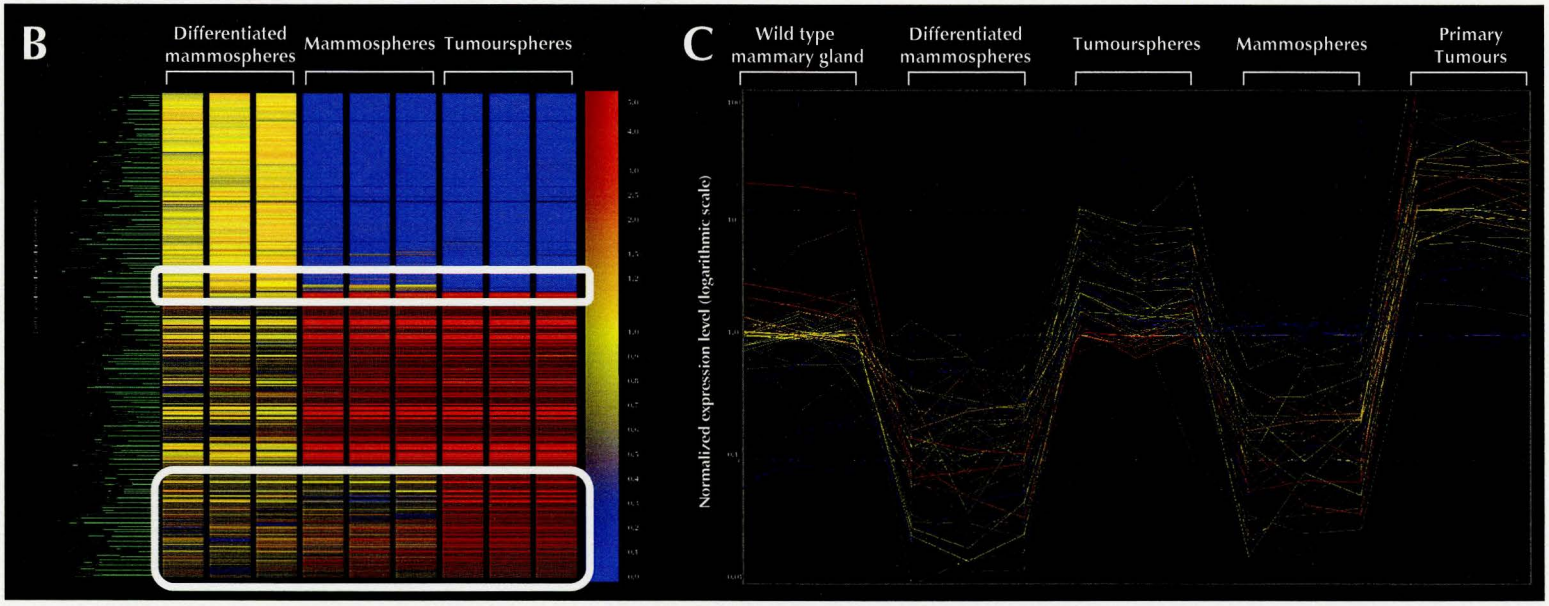


Figure 10. Tumourspheres express genes in common with primary Her2/Neu-induced mammary tumours: selection of *tumourness* genes. **(A)** Genes commonly preferential to mouse tumourspheres and two independent data sets from primary Her2/Neu-induced mammary tumours are potential biomarkers of the MMTV-Neu model of human breast cancer, and may also include candidate therapeutic targets. Genes preferentially expressed in tumourspheres **(grey)** are overlapped with Her2/Neu-induced primary tumours analyzed in our lab **(green)** and elsewhere **(red)**. The 68 genes in common represent a consensus set which identify Her2/Neu-induced tumour cells *in vitro* and *in vivo*. **(B)** Gene tree dendogram of all genes preferential to tumourspheres when compared with differentiated mammospheres. In these analyses, differentiated cells are used as a baseline (normalized expression level of 1.0 or 100%, heatmap yellow). Regions are marked where the expression profiles of mammospheres and differentiated mammospheres are similar but differ from that of tumourspheres. These genes were selected and filtered for preferential expression in primary tumours vs. the normal mammary gland. **(C)** The expression profile of tumourness genes across *in vitro* and *in vivo* mRNA samples deriving from normal mouse mammary tissue and Her2/Neu-induced mammary tumours.



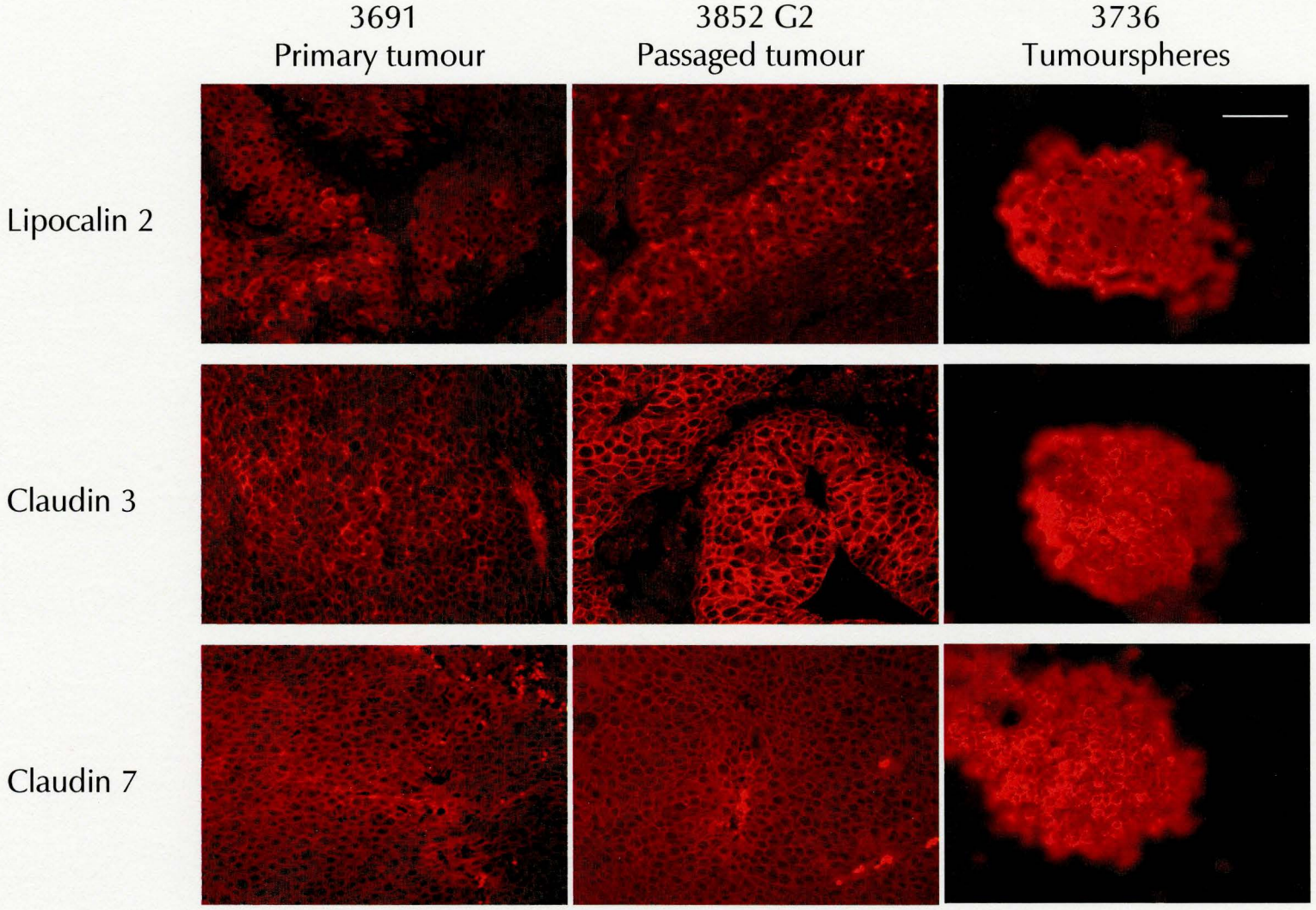


Figure 11. Her2/Neu-induced mouse mammary tumours express tumourness genes. Primary tumour 3691, serially passaged (generation 2) tumour 3852 and cultured tumourspheres deriving from tumour 3736 are stained with antibodies to Lipocalin2 **(top row)**, and tight junction proteins Claudin 3 **(middle row)** and Claudin 7 **(bottom row)**. Scale bar, 100μm.

Figure 12. Expression of Rat neu transgene in tumourspheres arising from MMTV-Neu primary tumours. Immunocytochemical analyses to rat Neu protein **(A)** and to phospho-Neu **(B)** shows staining of Her2 in tumoursphere line #3736. Merge with DAPI is inset in each panel. Cells stained with no primary antibody are included as a negative control (C red alone, D DAPI). Scale bar, 100 μm.

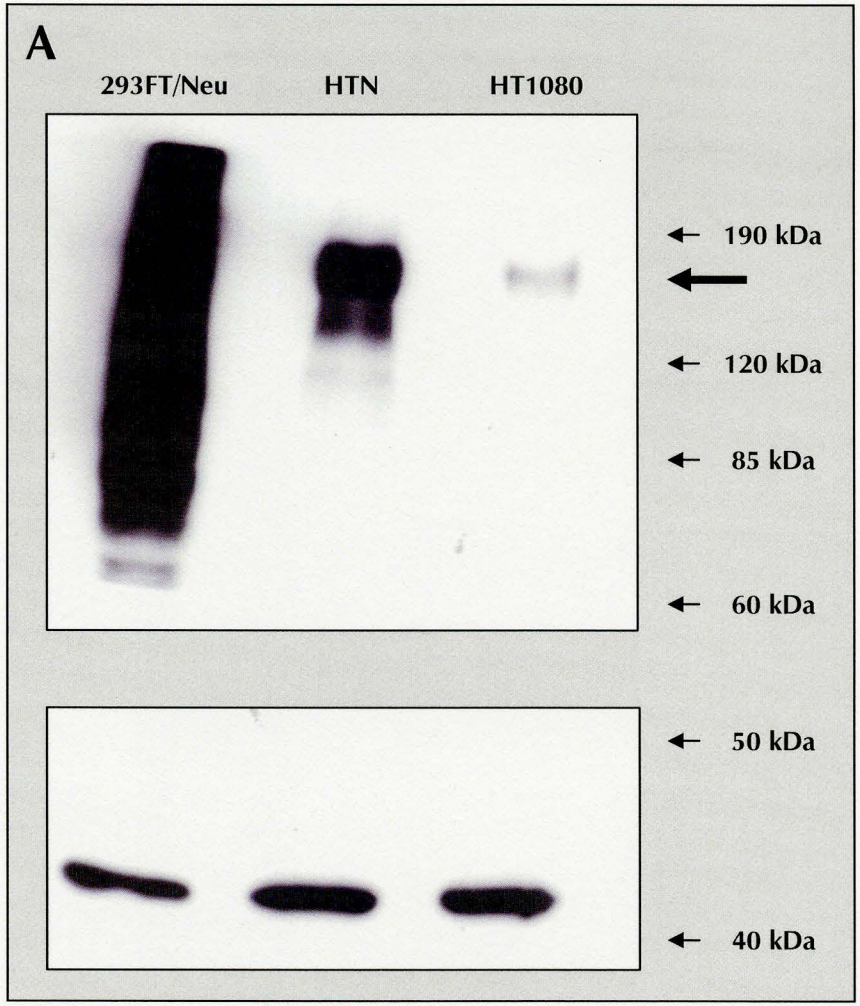


Figure 13 – Expression analyses of Her2/Neu protein in engineered human cell lines. (A) Whole cell lysates were prepared from 293FT cells transiently transfected with plasmid DNA encoding Rat neu cDNA (**293FT/Neu**), HT1080 cells transduced with a recombinant lentivirus encoding Rat neu cDNA (**HTN**) or wild-type HT1080 cells (**HT1080**, negative control). Protein was analyzed by western blot analysis as described in Materials and Methods with primary antibodies Ab-3 (mouse mAb, Rat/human reactive, Oncogene Science) and loading control β-actin (Abcam, 1:20,000). Endogenous expression of human erbB2/Her2/Neu protein is seen in the far right lane.

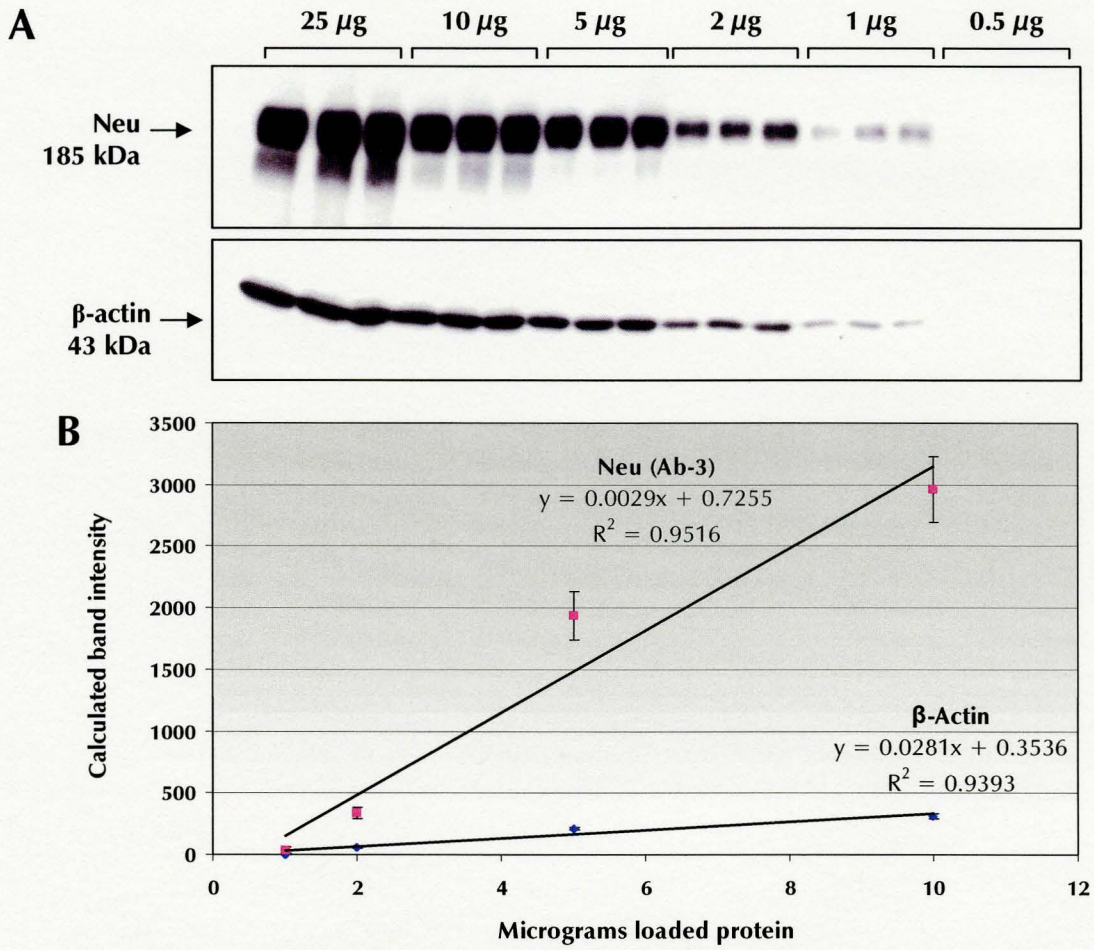
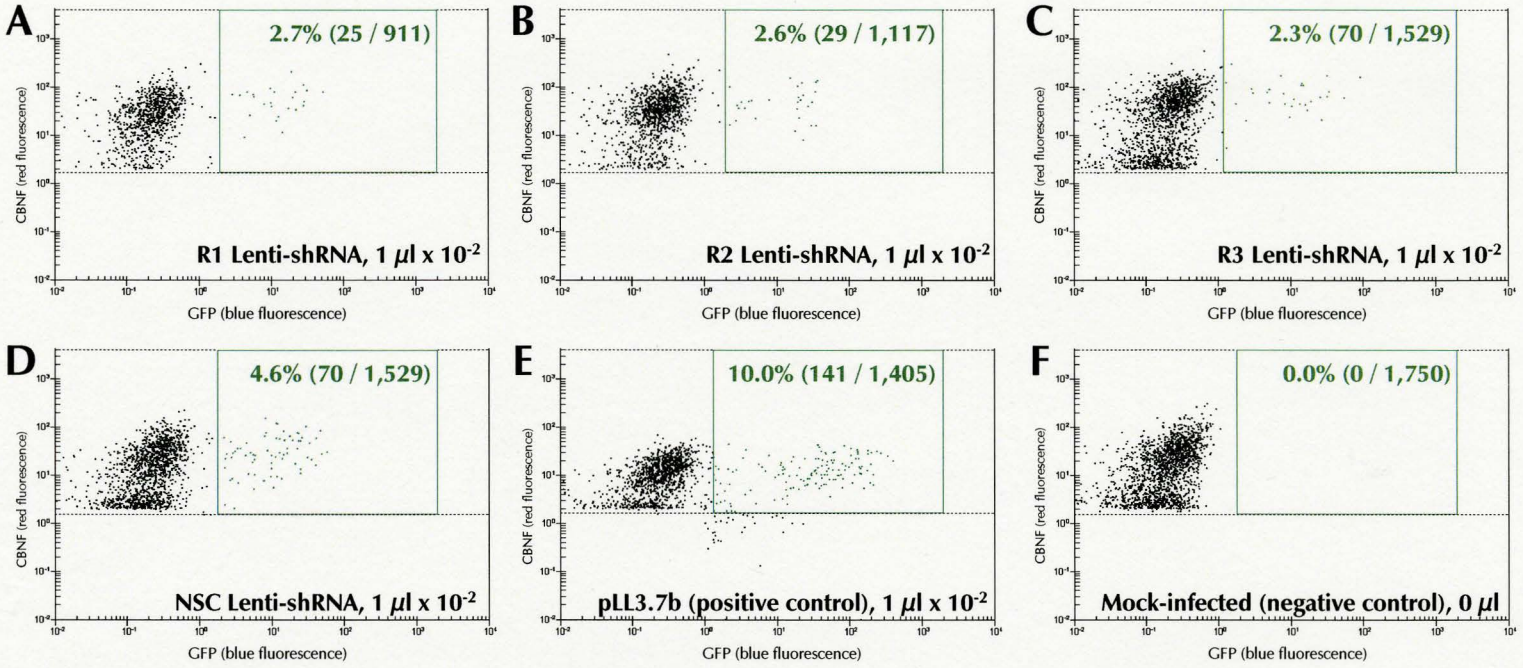


Figure 14 – Linear response titration of Neu$^+$ lysate. Whole cell lysates were prepared from HTN cells (HT1080 cells transduced with a recombinant lentivirus encoding Rat neu cDNA. Indicated quantities of total cell lysate were separated by SDS-PAGE (8%) as described previously. Membranes were incubated for 1 hour with primary antibodies Ab-3 and loading control β-actin (1:20,000 in 5% milk as described previously) and with goat anti-mouse IgG-HRP conjugate (Fab-specific, Sigma, 1:80,000) for 40 minutes at room temperature. Membranes were washed 3 times and exposed to film using ECL Advance reagents as per the manufacturer's instructions (**A**). Bands corresponding to Neu protein and β-actin were detected and quantified using Typhoon Trio (GE Healthcare) and ImageQuant software. (**B**) Calculated band densities for both Neu protein and β-actin are plotted versus micrograms of total lysate loaded for electrophoresis. Linear trendline reqression is reported with associated R-squared values for each. Samples corresponding to 25ug lysate were non-linear and were excluded from these analyses due to saturation of signal.



G

shRNA	Replicate		Percent positive			Viral titer			
	1	2	Average		St.Dev.	IU/μl	IU/ml		St.Dev.
R1	2.7%	2.7%	2.7%	±	0.00%	5.4E+05	5.4E+08	±	0.00E+00
R2	3.1%	2.6%	2.9%	±	0.35%	5.7E+05	5.7E+08	±	7.07E+07
R3	2.0%	1.6%	1.8%	±	0.28%	3.6E+05	3.6E+08	±	5.66E+07
NSC	4.6%	4.8%	4.7%	±	0.14%	9.4E+05	9.4E+08	±	2.83E+07
LL	10.3%	9.7%	10.0%	±	0.42%	2.0E+06	2.0E+09	±	8.49E+07

Figure 15 – Determination of lentiviral titer in HTN cells. Dilutions of concentrated lentivirus corresponding to 1 μL x 10^{-2}, 10^{-3}, and 10^{-4} were used to infect cells. Infective viral titer was determined 48 hours later as described by calculating the exact proportion of eGFP-positive cells in wells with approximately 1-10% of cells positive (**G**). Shown are Agilent Bioanalyzer flow cytometric dotplots from 2 X 10^5 HTN cells infected in duplicate with 1.0 x 10^{-2} μL of lentiviruses R1 (**A**), R2 (**B**), R3 (**C**), NSC (**D**), and pLL3.7b (**E**). Cells infected with LentiLox virus pLL3.7b (**E**) and those not infected with any virus (mock infected, **F**) were included as positive and negative controls, respectively. The percentage of eGFP positive cells and the corresponding event counts are shown for one of two infections performed and analyzed in parallel (**G**).

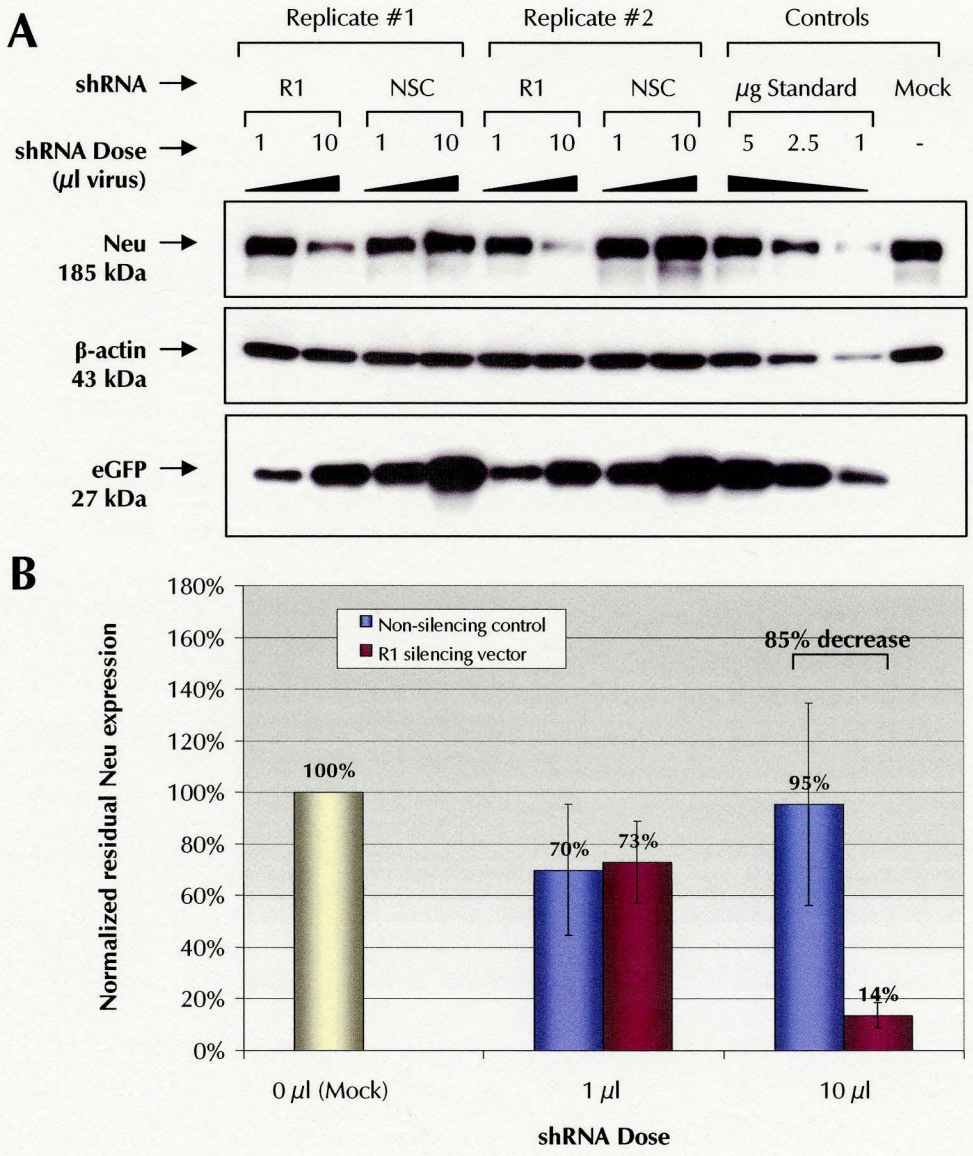


Figure 16. Silencing of Neu expression by Lenti-shRNA construct R1. HT1080 cells engineered to stably express the rat cDNA encoding Her2/Neu (HTN1 cells) were infected in duplicate wells of a 6 well dish with 0 µl (mock), 1 µl, or 10 µl doses of lentiviral shRNA **R1**. Viral titer of 2.7 x 10^8 TU/ml corresponds to MOI ~13 for 10 µl R1. A similar infection was carried out with a non-silencing (scrambled) shRNA lentiviral vector (NSC, 4.7 x 10^8 TU/ml) where 10 µl corresponded to MOI ~23 (**NSC**). Lysates were collected 8 days post-infection, and 10 µg were loaded per lane for separation by western blot **(A)**. A small standard curve of dilutions from the NSC 10 µl infection is included for comparison with Neu protein levels in samples infected with R1 silencing construct. The Neu protein levels in the 5, 2.5, and 1 µg standard curve lanes approximately correspond to 50%, 25%, and 10% residual Neu expression. **(B)** PVDF membranes were scanned with a Typhoon Trio and quantified using ImageQuant software. This experiment was performed in technical duplicate and was repeated in a second biological experiment with similar results.

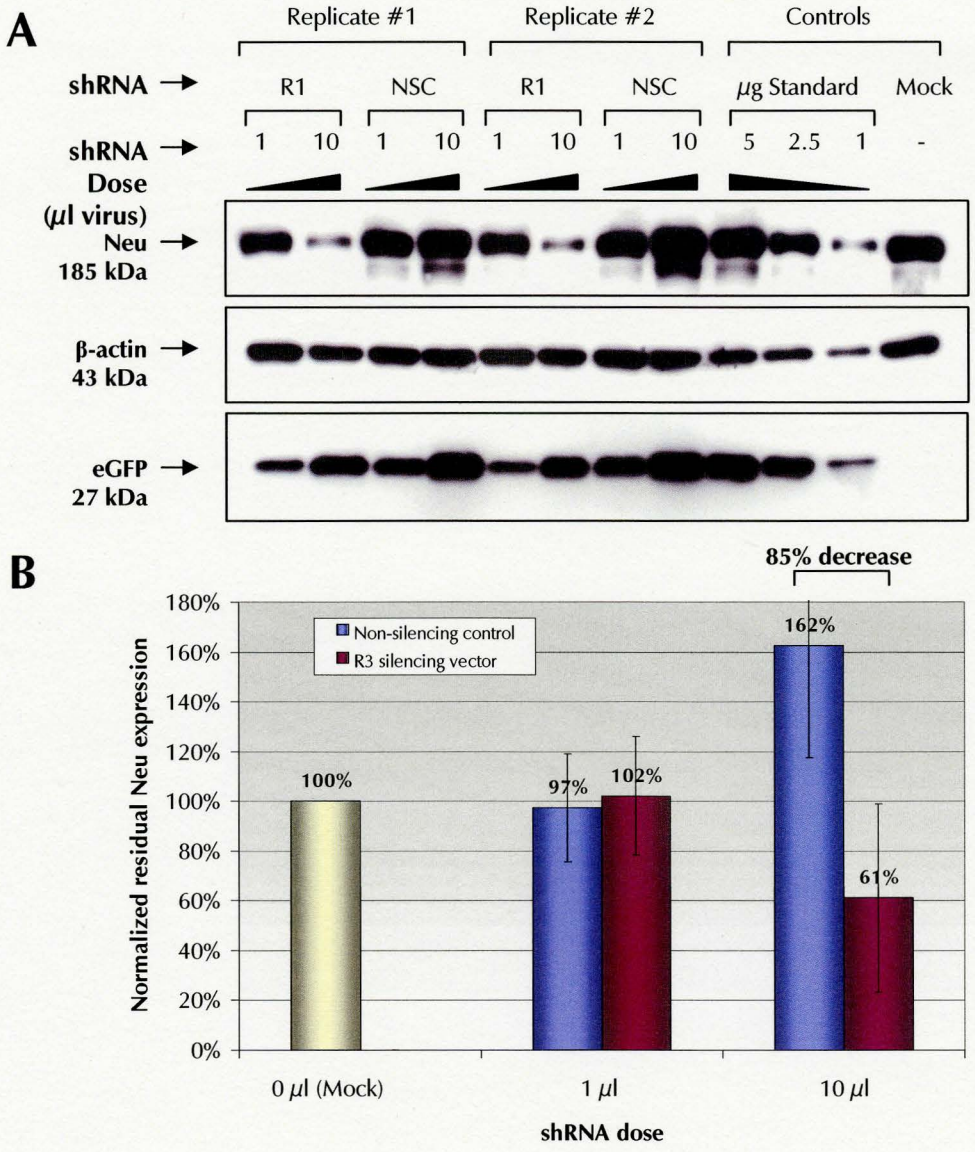


Figure 17. Silencing of Neu expression by Lenti-shRNA construct R1. HT1080 cells engineered to stably express the rat cDNA encoding Her2/Neu (HTN1 cells) were infected in duplicate wells of a 6 well dish with 0 μl (mock), 1 μl, or 10 μl doses of lentiviral shRNA **R1**. Viral titer of 2.7 x 10^8 TU/ml corresponds to MOI ~13 for 10 μl R1. A similar infection was carried out with a non-silencing (scrambled) shRNA lentiviral vector (NSC, 4.7 x 10^8 TU/ml) where 10 μl corresponded to MOI ~23 (**NSC**). Lysates were collected 8 days post-infection, and 10 μg were loaded per lane for separation by western blot **(A)**. A small standard curve of dilutions from the NSC 10 μl infection is included for comparison with Neu protein levels in samples infected with R1 silencing construct. The Neu protein levels in the 5, 2.5, and 1 μg standard curve lanes approximately correspond to 50%, 25%, and 10% residual Neu expression. **(B)** PVDF membranes were scanned with a Typhoon Trio and quantified using ImageQuant software. This experiment was performed in technical duplicate and was repeated in a second biological experiment with similar results.

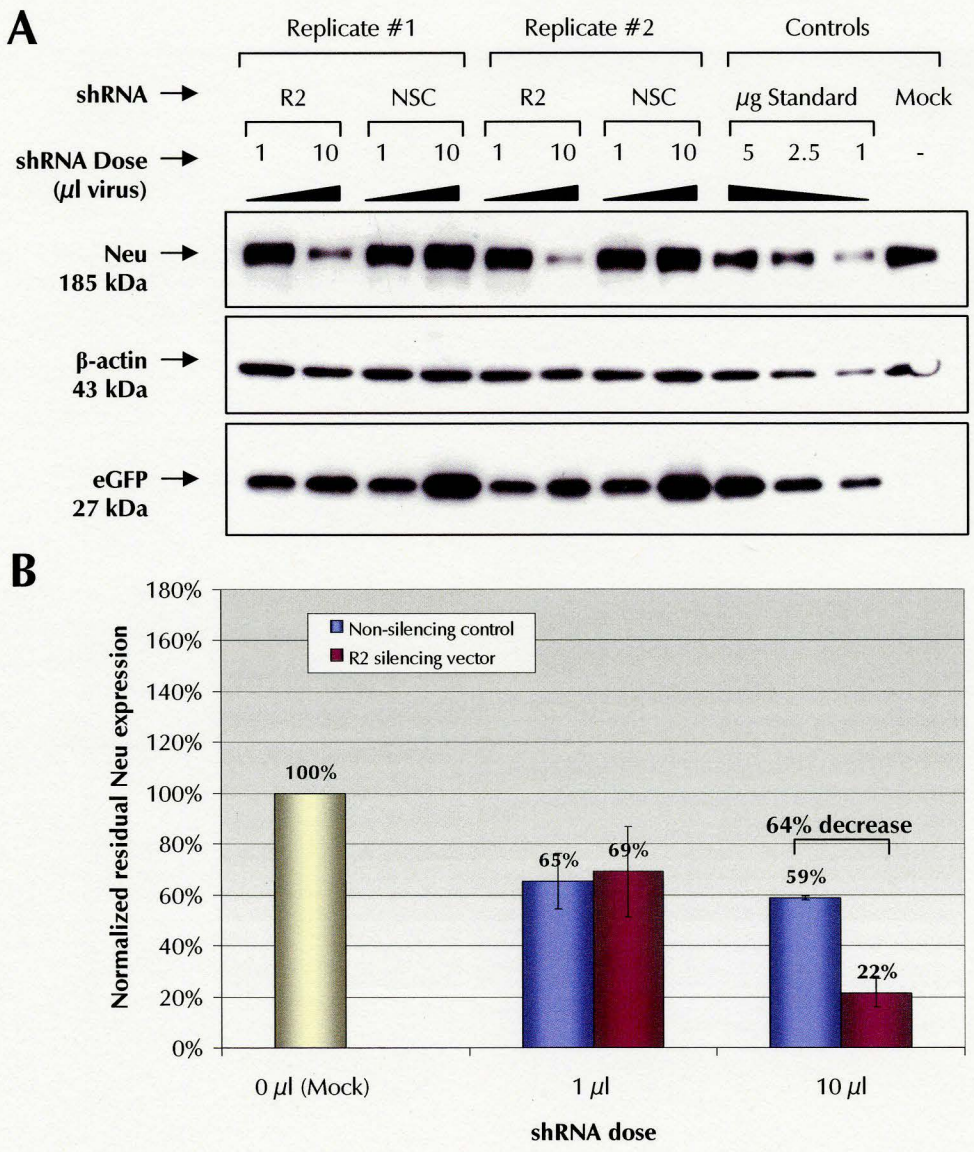


Figure 18. Silencing of Neu expression by Lenti-shRNA construct R2. HT1080 cells engineered to stably express the rat cDNA encoding Her2/Neu (HTN1 cells) were infected in duplicate wells of a 6 well dish with 0 μl (mock), 1 μl, or 10 μl doses of lentiviral shRNA **R2**. Viral titer of 3.2 x 10^8 TU/ml corresponds to MOI ~16 for 10 μl R2. A similar infection was carried out with a non-silencing (scrambled) shRNA lentiviral vector (NSC, 4.7 x 10^8 TU/ml) where 10 μl corresponded to MOI ~23 (**NSC**). Lysates were collected 8 days post-infection, and 10 μg were loaded per lane for separation by western blot **(A)**. A small standard curve of dilutions from the NSC 10 μl infection is included for comparison with Neu protein levels in samples infected with R2 silencing construct. The Neu protein levels in the 5, 2.5, and 1 μg standard curve lanes approximately correspond to 50%, 25%, and 10% residual Neu expression. **(B)** PVDF membranes were scanned with a Typhoon Trio and quantified using ImageQuant software. This experiment was performed in technical duplicate and was repeated in a second biological experiment with similar results.

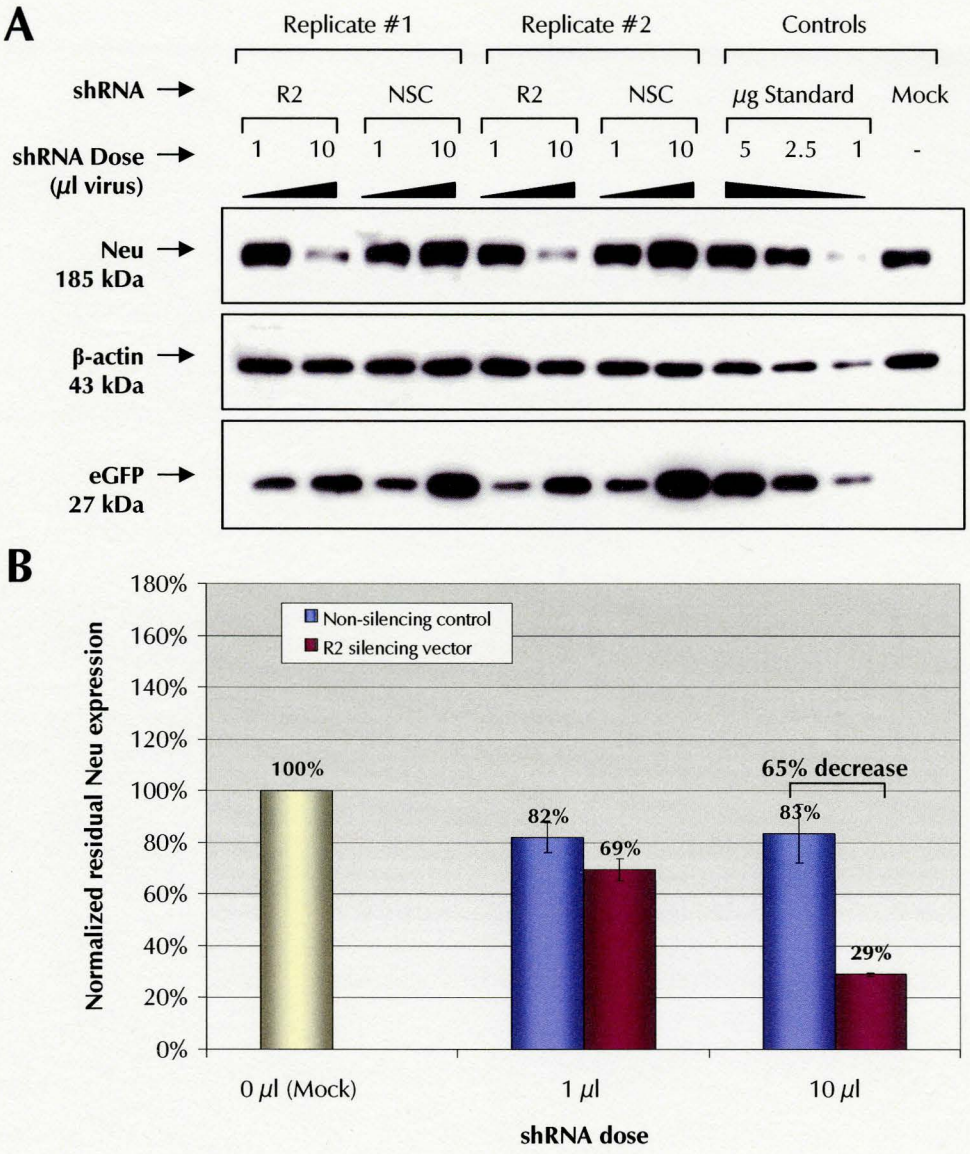


Figure 19. Silencing of Neu expression by Lenti-shRNA construct R2. HT1080 cells engineered to stably express the rat cDNA encoding Her2/Neu (HTN1 cells) were infected in duplicate wells of a 6 well dish with 0 μl (mock), 1 μl, or 10 μl doses of lentiviral shRNA **R2**. Viral titer of 3.2 x 10^8 TU/ml corresponds to MOI ~16 for 10 μl R2. A similar infection was carried out with a non-silencing (scrambled) shRNA lentiviral vector (NSC, 4.7 x 10^8 TU/ml) where 10 μl corresponded to MOI ~23 (**NSC**). Lysates were collected 8 days post-infection, and 10 μg were loaded per lane for separation by western blot **(A)**. A small standard curve of dilutions from the NSC 10 μl infection is included for comparison with Neu protein levels in samples infected with R2 silencing construct. The Neu protein levels in the 5, 2.5, and 1 μg standard curve lanes approximately correspond to 50%, 25%, and 10% residual Neu expression. **(B)** PVDF membranes were scanned with a Typhoon Trio and quantified using ImageQuant software. This experiment was performed in technical duplicate and was repeated in a second biological experiment with similar results.

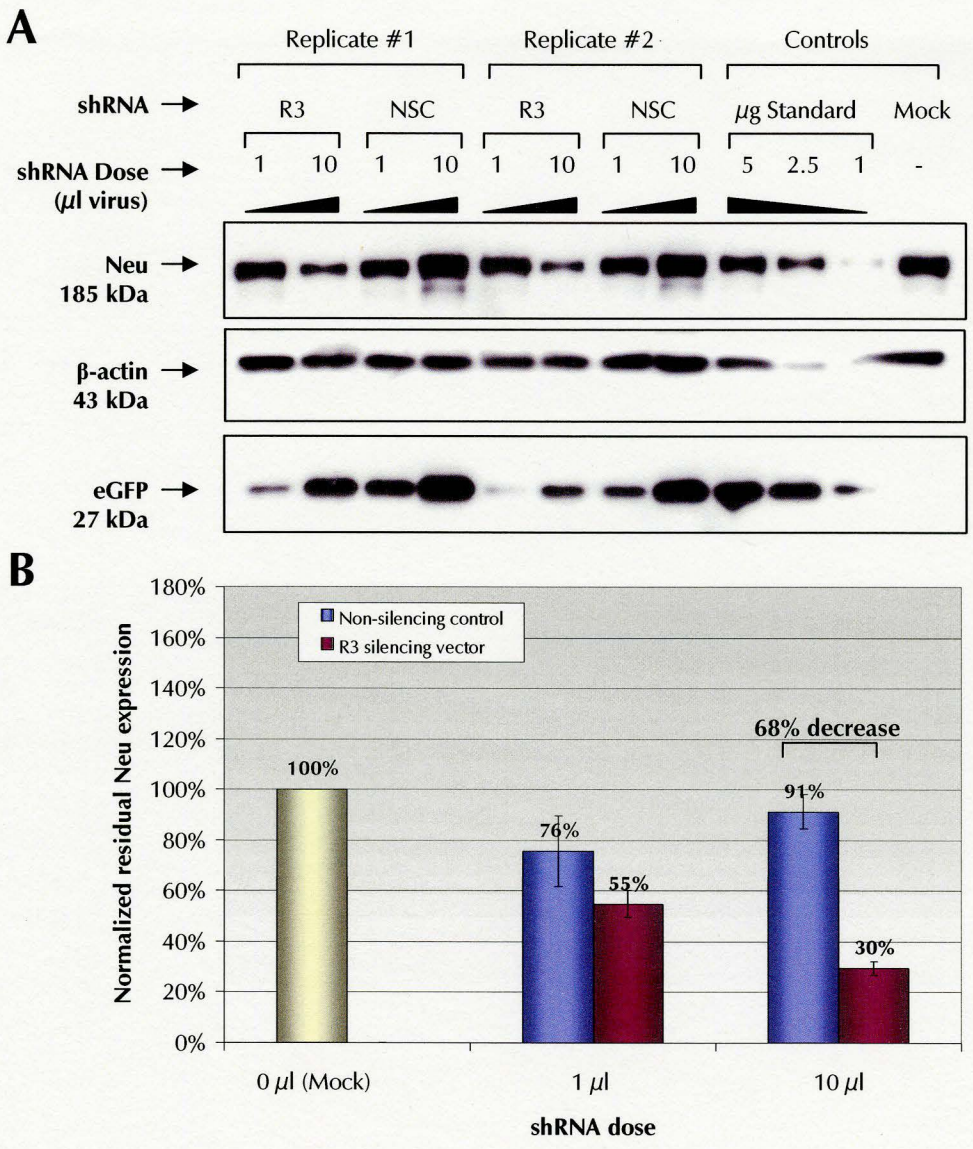


Figure 20. Silencing of Neu expression by Lenti-shRNA construct R3. HT1080 cells engineered to stably express the rat cDNA encoding Her2/Neu (HTN1 cells) were infected in duplicate wells of a 6 well dish with 0 μl (mock), 1 μl, or 10 μl doses of lentiviral shRNA **R3**. Viral titer of 1.8 x 10^8 TU/ml corresponds to MOI ~9 for 10 μl R3. A similar infection was carried out with a non-silencing (scrambled) shRNA lentiviral vector (NSC, 4.7 x 10^8 TU/ml) where 10 μl corresponded to MOI ~23 (**NSC**). Lysates were collected 8 days post-infection, and 10 μg were loaded per lane for separation by western blot **(A)**. A small standard curve of dilutions from the NSC 10 μl infection is included for comparison with Neu protein levels in samples infected with R3 silencing construct. The Neu protein levels in the 5, 2.5, and 1 μg standard curve lanes approximately correspond to 50%, 25%, and 10% residual Neu expression. **(B)** PVDF membranes were scanned with a Typhoon Trio and quantified using ImageQuant software. This experiment was performed in technical duplicate and was repeated in a second biological experiment with similar results.

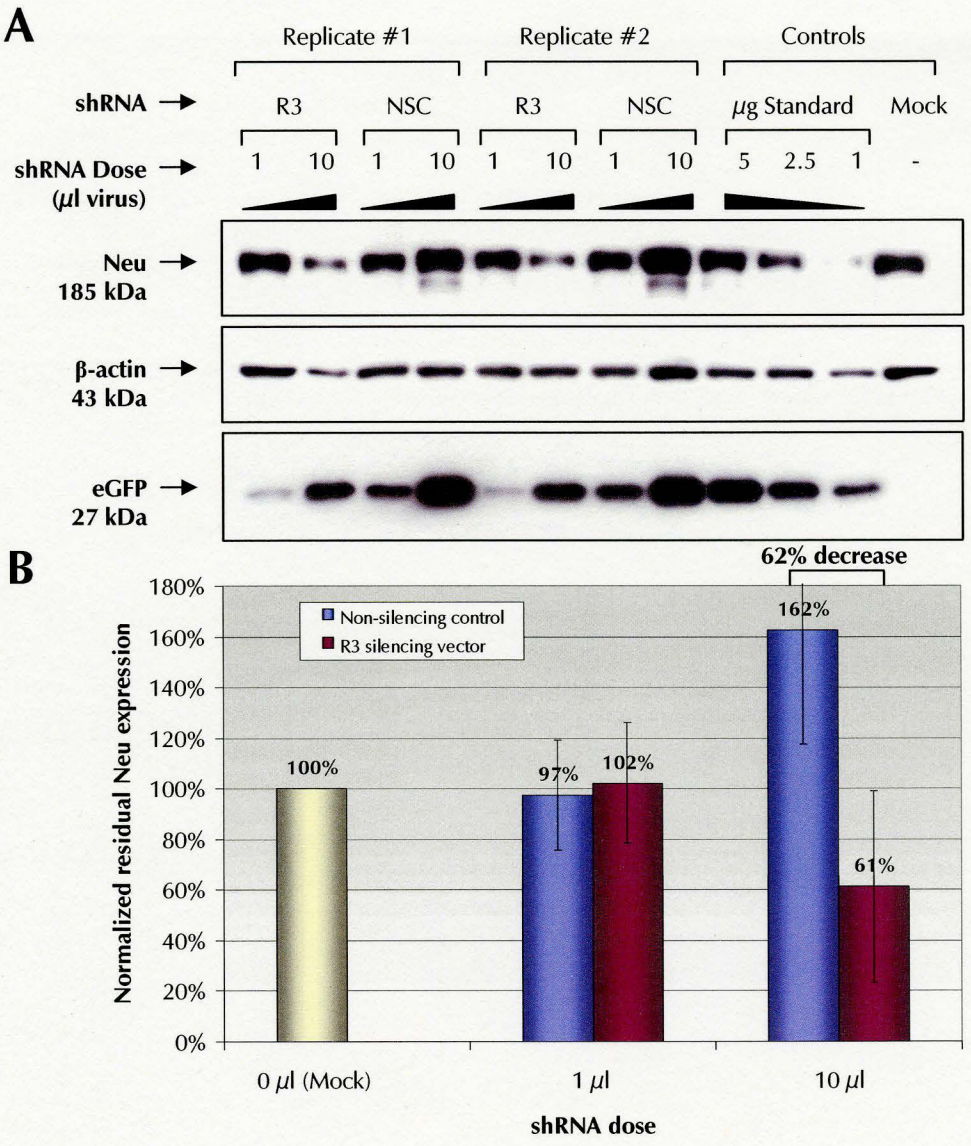
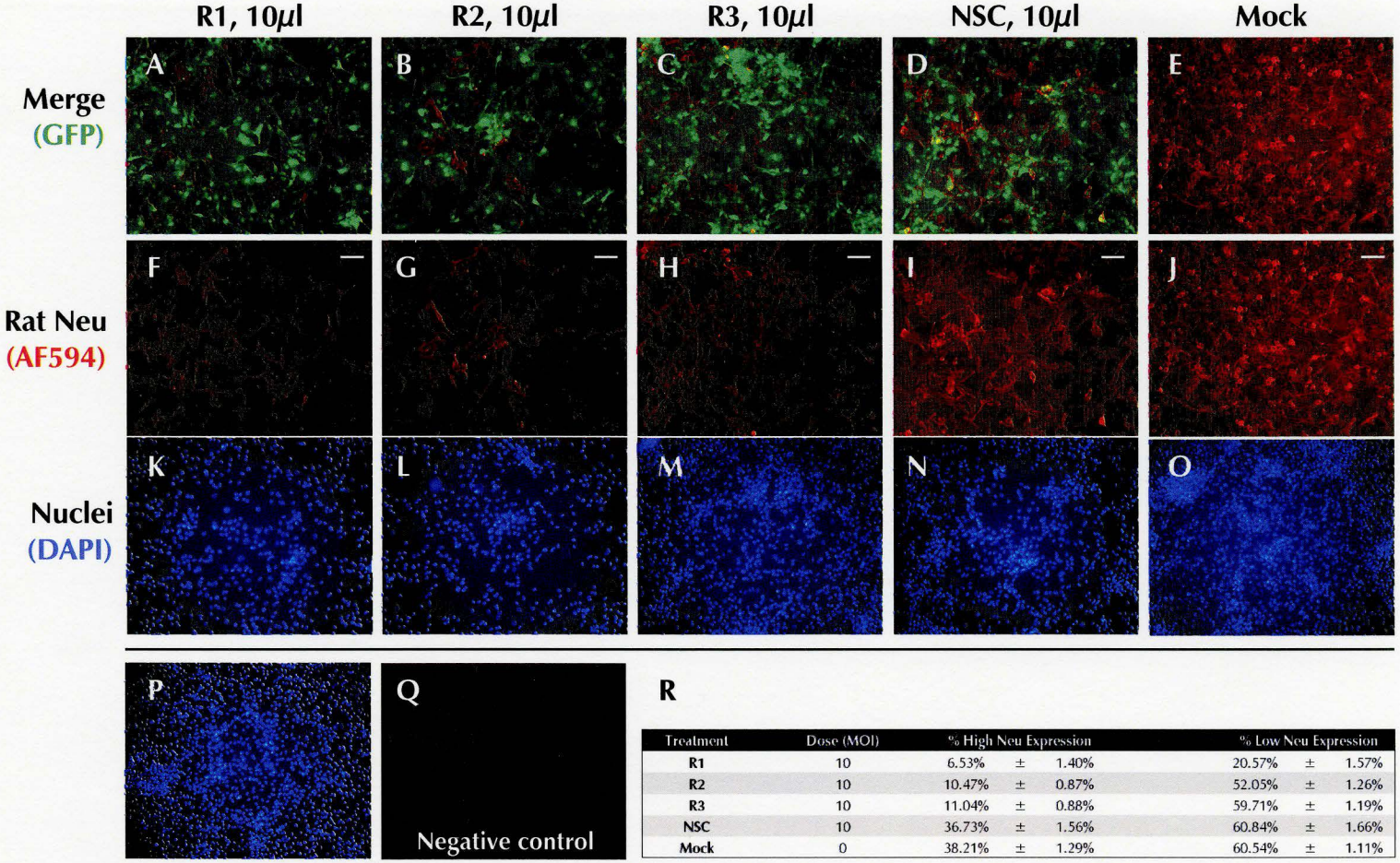


Figure 21. Silencing of Neu expression by Lenti-shRNA construct R3. HT1080 cells engineered to stably express the rat cDNA encoding Her2/Neu (HTN1 cells) were infected in duplicate wells of a 6 well dish with 0 μl (mock), 1 μl, or 10 μl doses of lentiviral shRNA **R3**. Viral titer of 1.8 x 10^8 TU/ml corresponds to MOI ~9 for 10 μl R3. A similar infection was carried out with a non-silencing (scrambled) shRNA lentiviral vector (NSC, 4.7 x 10^8 TU/ml) where 10 μl corresponded to MOI ~23 (**NSC**). Lysates were collected 8 days post-infection, and 10 μg were loaded per lane for separation by western blot **(A)**. A small standard curve of dilutions from the NSC 10 μl infection is included for comparison with Neu protein levels in samples infected with R3 silencing construct. The Neu protein levels in the 5, 2.5, and 1 μg standard curve lanes approximately correspond to 50%, 25%, and 10% residual Neu expression. **(B)** PVDF membranes were scanned with a Typhoon Trio and quantified using ImageQuant software. This experiment was performed in technical duplicate and was repeated in a second biological experiment with similar results.



R

Treatment	Dose (MOI)	% High Neu Expression	% Low Neu Expression
R1	10	6.53% ± 1.40%	20.57% ± 1.57%
R2	10	10.47% ± 0.87%	52.05% ± 1.26%
R3	10	11.04% ± 0.88%	59.71% ± 1.19%
NSC	10	36.73% ± 1.56%	60.84% ± 1.66%
Mock	0	38.21% ± 1.29%	60.54% ± 1.11%

Figure 22 – Neu expression in HTN cells following lenti-shRNA treatment. Lenti-shRNA viruses were used to infect HTN cells, and are marked with eGFP **(A-D)**, while no GFP signal is visible in mock-infected cells **(E)**. Compared with NSC-infected cells **(I)**, decreased Neu protein expression **(F, G, H)** is observed following treatment with indicated Lenti-shRNAs. The proportion of cells expressing high and low quantities was established in multiple random fields **(R)**. DAPI staining marks nuclei **(K-O).** Parental HT1080 cells stained with primary and secondary antibody serve as a negative control in these experiments **(P,Q)**. Scale bars, 100 μm.