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## Research Article

# Characterization *In vitro* and *In vivo* of Clonogenic Cells from Heterogeneous Cell Populations -

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## ABSTRACT

Normal tissues and tumors arise from a population of cells termed stem cells. In vivo experiments have provided evidence of the presence of stem cells throughout the mouse mammary gland. Premalignant mammary outgrowths that faithfully recapitulate the mammary epithelial cell lineage upon transplantation contain cells with tumor-forming potential. Cell sorting techniques have identified putative mouse mammary stem cell surface markers and human breast cancer stem cell surface markers. These markers do not identify only stem cells but in fact distinguish a mixed population of cells containing stem cell activity. Previous studies have demonstrated that clones arising from single cells in vitro can be categorized into three types based on the clone morphology. Here, we report the characterization, both in vitro and in vivo, of clonogenic cells from a non-tumorigenic mammary epithelial population and those from an erbB2-induced mammary tumor. We found that clones arising from normal mammary cells expressed different patterns of stem and developmental marker between the clone types and compared to the expression patterns observed on clones that developed from tumorigenic mammary cells.

**KEYWORDS:** Cancer; Clones; ErbB2; Mammary; Stem cells

## INTRODUCTION

Normal mammary development and homeostasis depends on the activity of subpopulations of stem cells and progenitor cells [1]. The presence of resident stem cells in the mouse mammary gland was demonstrated by the fact any portion of the mammary gland regardless of age of the donor animal can reconstitute a mammary outgrowth when transplanted into a juvenile female recipient whose mammary fat pad has been cleared of endogenous epithelium [2-4]. Somatic stem cells reside in the mammary gland during adulthood and are responsible for most of normal mammary development and homeostasis [5].

Within a tumor there are cells with the capacity to generate and maintain a new tumor upon transplantation [6]. These cells are termed cancer stem cells (CSCs), or tumor initiating cells, and are responsible for tumor initiation and progression and some cases tumor recurrence. CSCs are also believed to be resistant to most chemotherapies that target rapidly dividing cells [7-8]. CSCs, like normal stem cells, traverse the cell cycle more slowly and thus are not efficiently targeted by chemotherapeutic drugs [7-8].

Normal human keratinocytes and human carcinoma-derived cells generate a variety of in vitro colony forms that have been classified based on morphology [9-10]. The three morphological designations for clone type are holoclone, meroclone, and paraclone. Holoclones arise from stem and early progenitor cells and have a compact round morphology. Meroclones arise from early and late progenitors and have an intermediate phenotype. Paraclones arise from late progenitor and transit amplifying cells and form loose irregular colonies.

Here, we investigated whether cells from non-tumorigenic mouse mammary epithelial cells (COMMA-D) and cells derived from erbB2-overexpressing mammary tumors (MMTV-neu) are able to form similar colony types in vitro. Experiments where cells obtained from different clone types were transplanted as single cells in the epithelium-divested fat pads of Nu/Nu mice demonstrated the regenerative ability of the COMMA-D clone types to form non-tumorigenic mammary outgrowths and of the MMTV-neu clone types to form tumors.

## MATERIAL AND METHODS

### Cell lines and growth conditions

COMMA-D and COMMA-D $\beta$ geo cells were gifts from D.

Medina. The original COMMA-D cell line was established from normal mammary epithelial cells from a mid-pregnant Balb/c mouse [11-12]. The COMMA-D $\beta$ geo cell line constitutively expresses the *lacZ* reporter gene and are stably transfected with the dominant-selectable gene pSV2Neo that confers resistance to the antibiotic G418 [13]. Both COMMA-D lines were maintained in DMEM/F12 supplemented with 2% FBS (Atlanta Biologicals; Atlanta, GA), 1% antibiotic-antimycotic, insulin 10  $\mu$ g/ml, EGF 5 ng/ml and 0.01M HEPES (all media components except FBS from Life Technology, Grand Island, NY) at 37°C with 5% CO<sub>2</sub>.

The MMTV-neu cell line used in these experiments was isolated and established from a spontaneous mammary tumor that arose in a FVB female mouse carrying the MMTV-neu transgene [14]. The MMTV-neu line was maintained in DMEM containing 10% FBS and 1% antibiotic-antimycotic at 37°C with 5% CO<sub>2</sub>.

### Mammosphere culture conditions

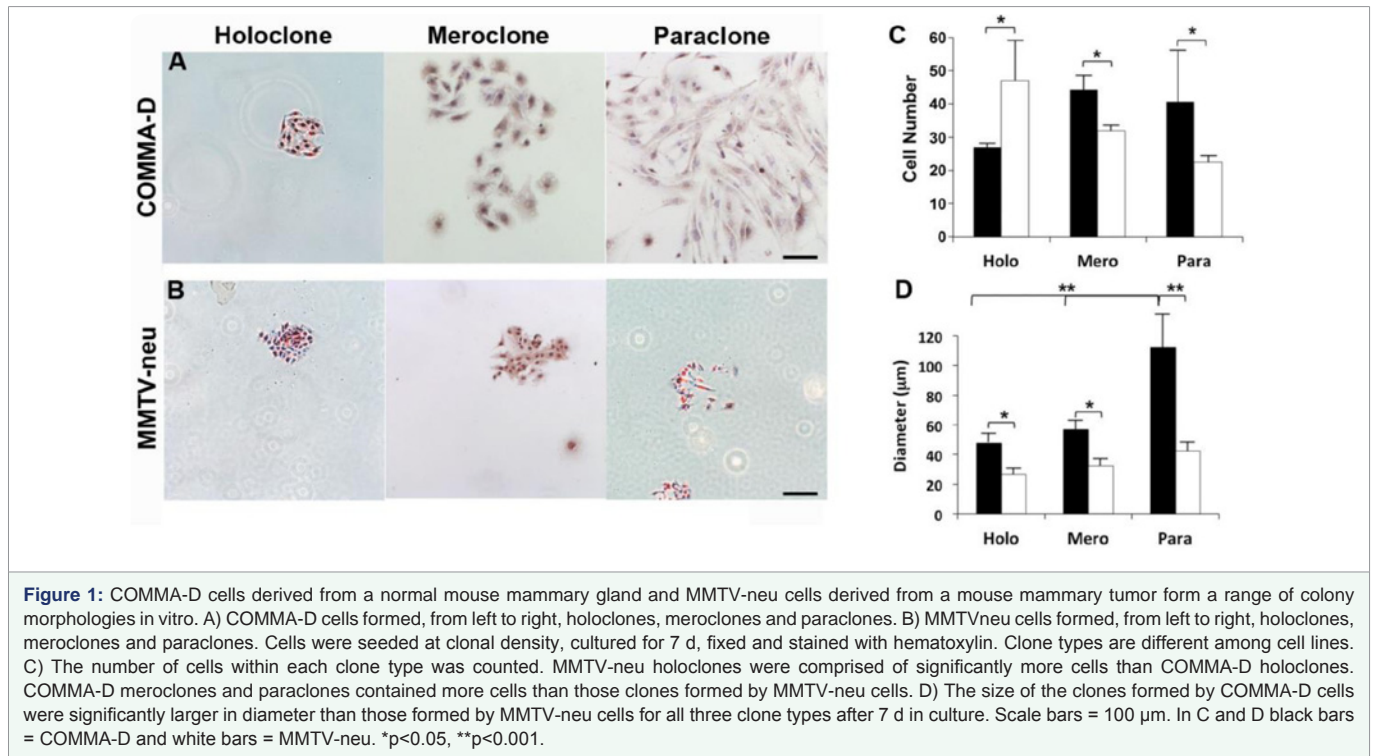
Cells were grown in non-adherent 3-dimensional culture as mammospheres based on previous published methods [15-16]. Briefly the cells were cultured in media comprised of a 3:1 mixture of DMEM and Ham's F-12 supplemented with basic fibroblast growth factor (bFGF) (20 ng/ml), epidermal growth factor (EGF) (20 ng/ml), heparin (4  $\mu$ g/ml; Sigma, St. Louis, MO) and B-27 (40  $\mu$ l of 50X stock/10 ml media; Sigma). The cells were seeded at 1000 cells/ml in ultra low attachment plates (Corning; Corning, NY) and maintained at 37°C with 5% CO<sub>2</sub>. Passaging of the cultures entailed collecting the media and non-adherent cells, centrifugation, trypsin treatment for 5 min and repeated pipetting to break up the spheres.

### Single cell cloning

To assess the ability of the different cell types within the heterogeneous COMMA-D cell line and the MMTV-neu cell line, single cells of each line were plated in 96-well plates and allowed to grow for 3 weeks. Colonies that developed were transferred to 12-well plates for further expansion and characterization. Following classification the clones were expanded further and cryopreserved for subsequent experiments.

### Immunohistochemistry

COMMA-D and MMTV-neu cultures were fixed with 4% paraformaldehyde and stained with hematoxylin (Sigma). For immunohistochemistry cultures were fixed as outlined above. Tissue sections were de-paraffinized using xylenes and graded ethanols. Endogenous peroxides were blocked with 0.3% H<sub>2</sub>O<sub>2</sub> for 10 min. Samples were washed with PBS then blocked with 10% serum in



PBS for 30 min at RT. Primary antibodies were added and incubated overnight at 4°C.

Primary antibodies used were anti-cytokeratins (Life Technology), anti-actin, anti-Notch1 (Santa Cruz Biotech; Santa Cruz, CA), anti-E-cadherin (R & D Systems; Minneapolis, MN), anti-CD24 (BD Pharmigen; San Jose, CA), anti-CD29 (BD Pharmigen), and anti-CD49f (BioLegend; San Diego, CA). Samples were washed 3x with PBS and secondary HRP-conjugated antibodies were added for 45 min at RT. Samples were washed 3x with PBS then exposed to DAB (Life Technology) for 10 min at RT. Sections were rinsed with PBS, washed with diH<sub>2</sub>O then counterstained with hematoxylin (Life Technology). Samples were dehydrated through graded ethanols and xylenes then coverslipped using Permount.

### Tissue transplantation

Transplantation of spheres into the mammary fat pad of Nu/Nu mice was executed following the fragment transplantation technique described by DeOme and colleagues [2]. Briefly, following the removal of epithelium of the #4 mammary fat pads, COMMA-D or MMTV-neu clones suspended in 10 μl of media were injected. Dispersed cells were resuspended in 10 μl of DMEM media and injected in the fat pads of Nu/Nu mice as described above. Fat pads transplanted with COMMA-D mammospheres were harvested 12 weeks from injection. Mammary outgrowths were prepared as whole mounts in which the entire mammary outgrowth was spread on a glass slide and fixed for 1-2 hrs in 4% paraformaldehyde in PBS. Tissues were washed repeatedly in PBS and processed for X-gal [17-18]. Following X-gal staining the whole mounts were embedded in paraffin and sectioned. Tumors that arose in fat pads transplanted with MMTV-neu spheres were removed once they reached 1 mm<sup>3</sup>. The tumors were cut in half with one half fixed and embedded in paraffin and the other half used for dissociation and propagation. Briefly, tumor halves were either minced and plated or incubated overnight in 1 mg/ml type I collagenase in DMEM with 5% FBS. Cells were triturated,

washed with PBS and plated in DMEM containing 10% FBS and 1% antibiotic-antimycotic.

All mice were housed in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

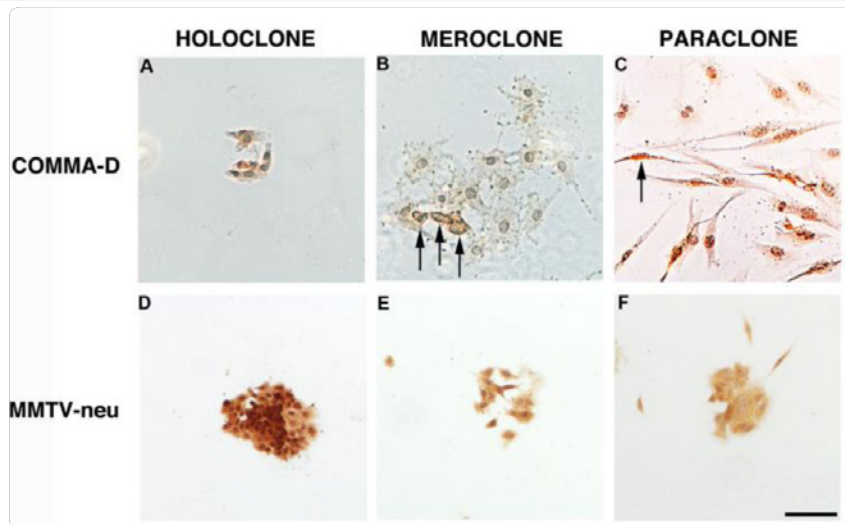
## RESULTS

### Colony morphologies

When seeded at low densities (1 cell/well) COMMA-D cells and MMTV-neu cells formed colonies after 2-3 days. The colonies were classified as holoclones, meroclones or paraclones based on morphology (Figure.1) [9-10]. The COMMA-D cells formed all three types of clones readily (Figure.1a). The MMTV-neu cells formed holoclones and meroclones but the formation of paraclones by the MMTV-neu cells was rare, less than 1 paraclone formed from 500 cells (Figure.1b).

After 7 days in culture colonies were imaged and the colony sizes were calculated using Zeiss software. The number of cells comprising each clone type were manually counted and found to be significantly different between the two cell lines (Figure.1c). Colonies formed by the normal COMMA-D cells were significantly larger in diameter than corresponding clone type of MMTV-neu cells (Figure.1d). The same number of cells was seeded for both cell lines. However in these experiments we cannot preclude more than one cell attaching and proliferating to form a colony.

The paraclones formed by COMMA-D cells were significantly wider than any holoclones or meroclones formed by the COMMA-D cells. The MMTV-neu cancer cell line formed holoclones comprised of significantly more cells than holoclones formed by the COMMA-D cells. However, the meroclones and paraclones formed by the COMMA-D cells contained more cells than the corresponding clones formed by the MMTV-neu cells. Holoclones, and to a lesser



**Figure 2:** Clone types express different levels of Notch1. COMMA-D A) holoclone, B) meroclone and C) paraclone express different levels of Notch1. MMTV-neu D) holoclone, E) meroclone, and F) paraclone all express Notch1. Arrows indicate examples of cells expressing Notch1.

extant meroclones, are comprised of cells growing in compact groups while the space between cells in paraclones is greater (Figure.1). The COMMA-D cell line was established from normal mammary epithelial cells from a mid-pregnant Balb/c mouse [11] while the MMTV-neu cell line we use is derived from the FVB background [14]. In order to preclude the possibility that differences in colony formation between the two lines is due to the different genetic background primary mammary epithelial cells from virgin Balb/c and FVB females were seeded in clone forming conditions. No differences in clone formation were found between the two genetic backgrounds (data not shown).

### Colony characterization

In order to further characterize the different clone types formed by the COMMA-D cells and MMTV-neu cells, clones were fixed and stained with a number of different antibodies specific for structural proteins and epithelial differentiation markers. Expression of actin by the various clone types was investigated (Supplemental Figure.1). Within the COMMA-D clones, the highest expression of actin was found in paraclones (Supplemental Figure.1c). Given the morphology of paraclones, comprised of elongated cells, this is expected. In contrast to the COMMA-D clones, MMTV-neu clones expressed high levels of actin (Supplemental Figure.1d-f).

E-cadherin is expressed on all mammary cells and has been identified as a tumor suppressor gene that is modified in many types of cancer [19-20]. Loss of E-cadherin expression is a hallmark of epithelial-mesenchymal transition (EMT) and immunohistochemical staining of E-cadherin is used to differentiate between lobular and ductal lesions [21]. Numerous cells within COMMA-D holoclones expressed E-cadherin (Supplemental Figure.2a). Fewer cells express E-cadherin in COMMA-D meroclones and even fewer in COMMA-D paraclones (Supplemental Figure.2b-c). E-cadherin was highly expressed in MMTV-neu holoclones and meroclones and was detected in the limited number of MMTV-neu paraclones that formed (Supplemental Figure.2d-f).

Notch1 is an important regulator of mammary epithelial stem and progenitor cells [22-23]. Notch1 was found expressed by the majority

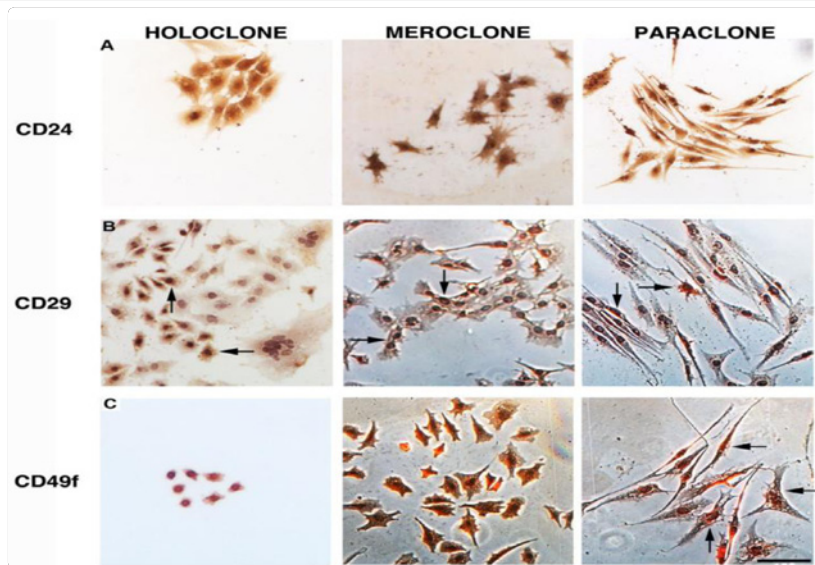
of cells within COMMA-D holoclones (Figure.2a). However, the expression of Notch1 in COMMA-D meroclones and paraclones was restricted to individual cells or clusters of cells within the clone outgrowths (Figure.2b-c). Arrows in (Figure. 2b) indicate examples of cells expressing Notch1 within the meroclone. This observation suggests that within the clone outgrowths different degrees of differentiation are present. We have previously demonstrated that Notch1 regulates asymmetric division in normal mammary epithelial cells, a function of stem and progenitor cells [22]. Expression of Notch1 was found in all cells comprising the different clone types formed by MMTV-neu cells (Figure.2e-f) with the highest expression in holoclones.

CD24, CD29 and CD49f have been established as cell surface markers of mouse mammary progenitor cells based on cell sorting, *in vitro* colony forming assays, and transplantation studies [23-24]. These markers have been used to sort out cancer stem cells from mouse mammary tumors [25-27]. Immunohistochemistry using anti-CD24 showed that the majority of cells within the three clone types formed by COMMA-D cells express CD24 (Figure.3a). Similar results were observed using anti-CD49f where all cells within COMMA-D holoclones and meroclones express CD49f (Figure.3c). However, expression of CD49f within COMMA-D paraclones was not uniform (Figure.3c). Expression of CD29 expression in all three types of COMMA-D clones was detected only in individual cells within clone (Figure.3b, arrows).

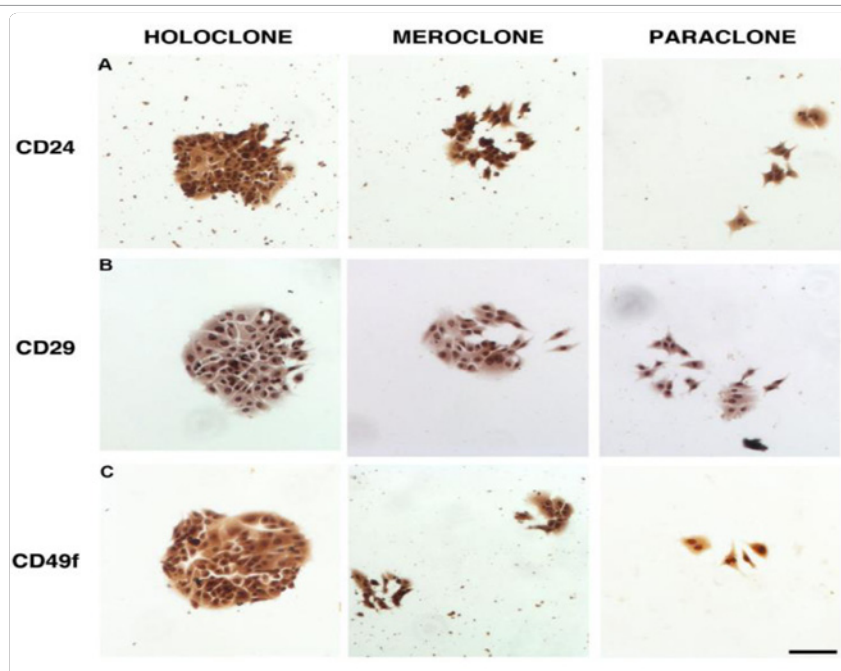
All cells within all three clone types formed by MMTV-neu cells express CD24 and CD49f (Figure.4a-c). Unlike the COMMA-D clones, no CD29 expression was detected in any cell making up the MMTV-neu clones (Figure.4b).

### Clone types form floating spheres

The free-floating mammosphere culture system has been established as an assay used to investigate stem cell behavior *in vitro* [15]. We have previously demonstrated that the COMMA-D cell line forms mammospheres [16]. Individual clones that arose from the COMMA-D and MMTV-neu cell lines were expanded then seeded in mammosphere forming conditions. No MMTV-neu paraclones expanded, 4-5 days after the first passage all paraclone cells were



**Figure 3:** COMMA-D clones express normal stem cell markers. COMMA-D clones were stained with antibodies specific for normal mammary stem cell markers. COMMA-D clones express A) CD24, B) CD29, and CD49f. Scale bar = 100  $\mu$ m. Arrows in B indicate examples of individual cells expressing CD29 in B or CD49f in C.



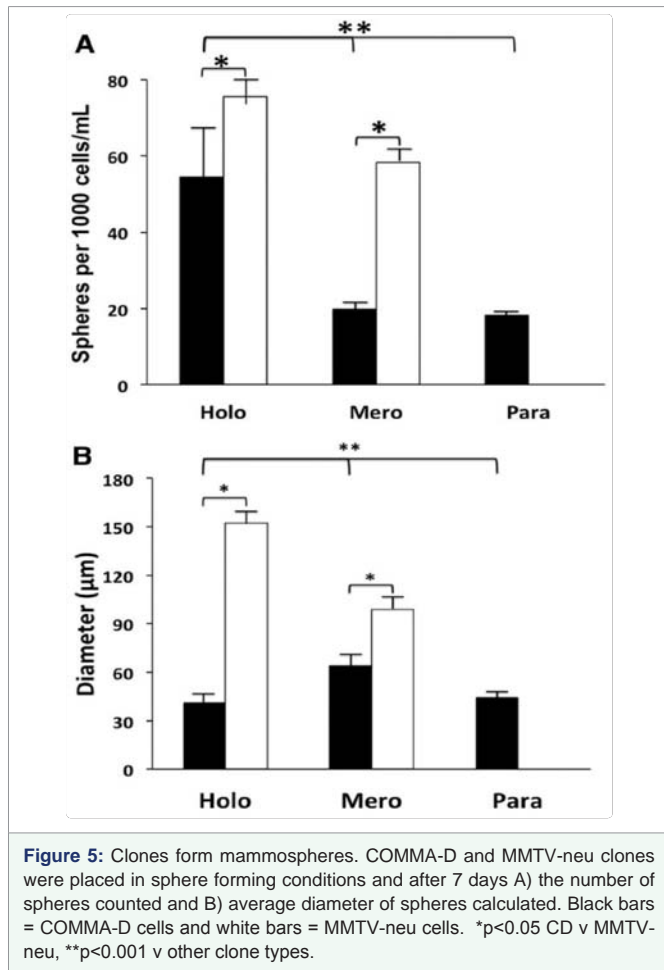
**Figure 4:** MMTV-neu clones express some normal mammary stem cell markers. MMTV-neu clones were stained antibodies specific for normal mammary stem cell markers. MMTV-neu clones express A) CD24, B) CD29, and CD49f. Scale bar = 100  $\mu$ m.

dead. The number of mammospheres formed was determined after 7 days in sphere forming conditions. Cells derived from holoclones formed a significant greater number of mammospheres than cells derived from meroclones or paraclones for both cell lines (Figure.5a). Cells derived from MMTV-neu holoclones and meroclones had a higher sphere forming efficiency than COMMA-D cells derived from holoclones and meroclones. The diameters of the mammospheres were also measured (Figure.5b). Spheres that formed from MMTV-neu holoclones and meroclones were significantly larger than the spheres formed by COMMA-D cells. The mammospheres formed by COMMA-D meroclones were larger than the mammospheres

formed by COMMA-D holoclones and paraclones.

### In vivo activity of clone types

When transplanted into mammary fat pads cleared of endogenous epithelium of juvenile mice the COMMA-D cell line is able to recapitulate a mammary outgrowth [11,16,29-30]. When transplanted in a similar manner MMTV-neu cells form mammary tumors [31,32]. Each clone type from both cell lines, with the exception of MMTV-neu paraclones as these clones could not be expanded, were transplanted into recipient juvenile immunocompromised female mice (Table 1). Mammary outgrowths formed by the COMMA-D clones were



harvested after 12 weeks. COMMA-D meroclones were able to form ductal and alveolar structures (Figure.6a-b). In vivo growth of COMMA-D holoclones and paraclones was not as robust as the meroclones. The COMMA-D holoclones and paraclones produced small outgrowths after 12 weeks, neither producing mammary outgrowths that filled the mammary fat pad (Supplemental Fig 3).

MMTV-neu meroclones formed mammary tumors within 5 weeks of transplantation while the MMTV-neu holoclones formed tumors within 10 weeks (Table 1). A cross section of a mammary tumor formed by an MMTV-neu meroclone is shown (Figure.6c). Both MMTV-neu clone types were 100% efficient at forming tumors although the MMTV-neu holoclones formed tumors slower than meroclones (Table 1).

Portions of the mammary tumors formed by MMTV-neu holoclones and meroclones were dissociated and expanded in culture. The cells isolated from this expansion were seeded in low densities to investigate whether the tumor cells were able to form the initial clone types. Dissociated tumor cells from an MMTV-neu meroclone mammary tumor were able to form all three clone types, holoclones (Figure.7a), meroclones (Figure.7b), and paraclones (Figure.7c). Similar results were observed with clones that arose from cells dissociated from a mammary tumor formed by an MMTV-neu holoclone. Holoclones (Figure.7d), meroclones (Figure.7e) and paraclones (Figure.7f) were observed.

## DISCUSSION

The COMMA-D cell line was established from the normal

mammary gland of a mid-pregnant mouse [11-12]. The COMMA-Dβgeo cell line is a derivative of the COMMA-D line that was infected with a retrovirus expressing a neomycin-resistance gene and β-galactosidase [13]. Comma-Dβgeo is a heterogeneous cell line that is able to reconstitute mammary glands upon transplantation thus maintaining stem/progenitor cell qualities. Numerous studies have identified cell surface markers of mammary stem cells such as CD24, CD29 and CD49f [23,24]. A human breast cancer stem cell expressing a CD24/CD44<sup>+</sup> surface marker phenotype has also been identified [33]. The normal mouse mammary stem cell markers have also been used to isolate cancer stem cells from mammary tumors [25-28]. Our results demonstrate that all cells in COMMA-D clones express CD24 and CD49f (Figure. 4A and C) but CD29 expression is limited in COMMA-D meroclones and paraclones. All MMTV-neu clones express CD24 and CD49f but no MMTV-neu clones express CD29.

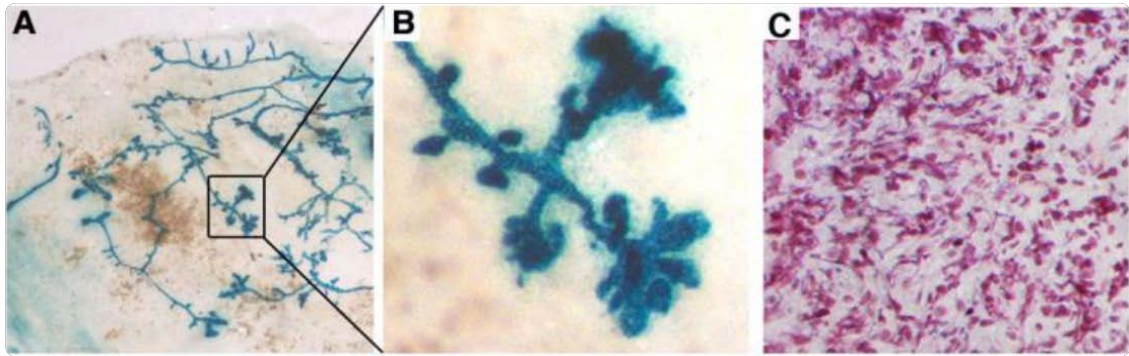
MMTV-neu holoclones and meroclones express Notch1 although expression is highest in holoclones. All cells in COMMA-D holoclones express Notch1 but expression is limited in meroclones and paraclones. Expression of Notch1 is associated with stem cells function, both normal and cancer [34,35], and is required for asymmetric division in normal mammary stem cells [22]. The absence of Notch1 in MMTV-neu paraclones compared to holoclones may be a reason that the clones do not expand as the stem cell component is lost. Similar reasoning can be applied to the fact that COMMA-D paraclones do not form mammary outgrowths in vivo.

MMTV-neu cells generally did not form paraclones in these experiments. Mammary tumors that form in MMTV-neu mice are estrogen receptor (ER)-negative and progesterone receptor (PR) negative indicating that the capacity for differentiation in these cells is limited [32]. Paraclones are believed to form from late-stage progenitor and transit amplifying cells therefore this result is to be expected. MMTV-neu targets the lobule-limited progenitor cells of the mouse mammary gland for transformation [36-38]. The lobule-limited progenitors, also known as parity-identified mammary epithelial cells (PI-MECs), are semi-differentiated cells that do not express ER or PR. They are not true multi-potent progenitor cells, rather cells that differentiate into milk protein producing cells during pregnancy. Paraclones represent a more differentiated phenotype than holoclones and meroclones. Since MMTV-neu targets a less differentiated cell type (PI-MECs), and expands this population, it stands to reason that MMTV-neu cells formed less paraclones.

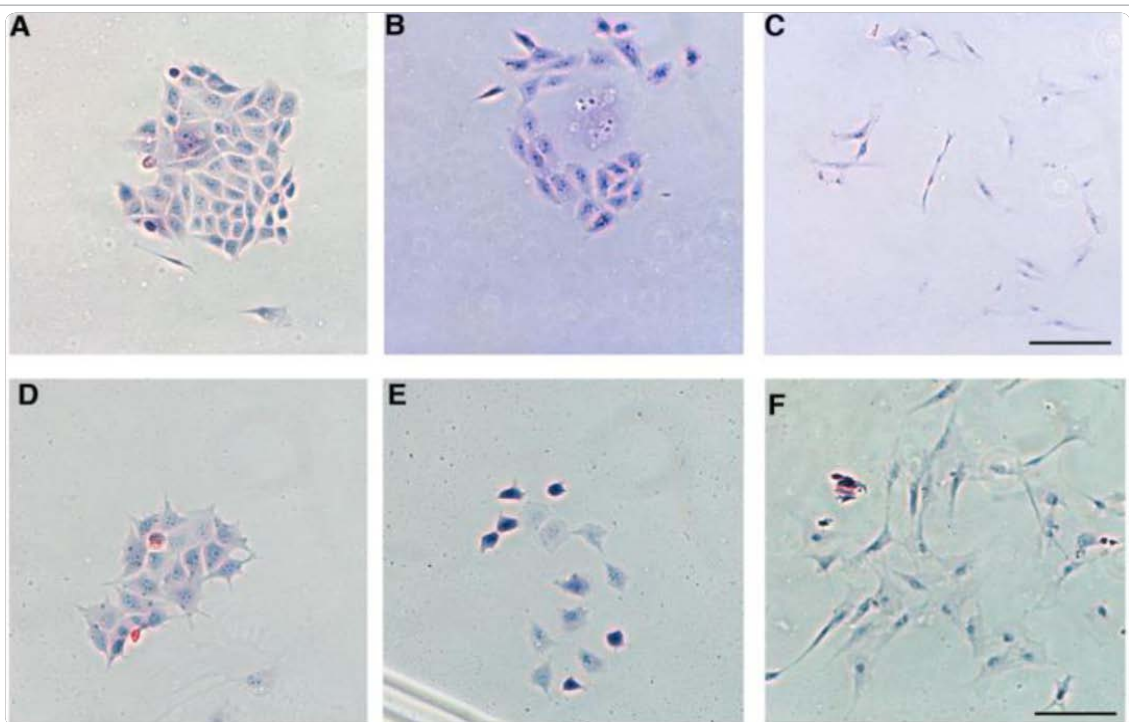
Paraclones have limited growth potential and form terminal colonies [9]. The established MMTV-neu cell line formed very few paraclones. However numerous paraclones were formed by cells freshly dissociated from MMTV-neu tumors formed by holoclones and meroclones. The established MMTV-neu cell line has undergone numerous cell divisions and passages in vitro while the freshly isolated tumor cells have undergone limited passaging in vitro. It may be that after numerous passages the newly formed MMTV-neu cell

**Table 1:** Transplantation results.

	Normal Dev	Tumor Dev
MMTV-neu holo	0/2	2/2
MMTV-neu mero	0/2	2/2
COMMA-D holo	2/4	0/4
COMMA-D mero	4/4	0/4
COMMA-D para	2/4	0/4



**Figure 6:** In vivo activity of clone types. COMMA-D and MMTV-neu clones were transplanted into cleared mammary fat pads of female juvenile mice. A) COMMA-Dβgeo meroclone formed mammary outgrowth. B) Higher magnification of box in (A). MMTV-neu meroclone formed a mammary tumor when transplanted. (A and B) Xgal stained, (C) stained with hematoxylin.



**Figure 7:** Dissociated MMTV-neu tumor cells form different clone types. Mammary tumors formed by MMTV-neu clone types were dissociated. Cell derived from a mammary tumor formed by an MMTV-neu meroclone formed A) holoclones, B) meroclones and C) paraclones. Cells dissociated from an MMTV-neu holoclone formed D) holoclones, E) meroclones and F) paraclones. Scale bar = 100 μm.

lines may also have a decreased capacity to form paraclones as the more differentiated cells die off in culture. Our results suggest that in vivo and in vitro conditions select against the expansion of the more differentiated MMTV-neu cells that give rise to paraclones.

COMMA-D meroclones are the only COMMA-D clone type that retains mammary repopulation capacity in vivo while COMMA-D holoclones produced larger mammospheres. This is perhaps due to the heterogeneous nature of the meroclone. Meroclones are intermediate clones, between holoclones and paraclones, and therefore contain multiple cell types. The multiple cell types allow for an increased capacity to form stem cell niches when transplanted. Stem cells rely on biochemical and physical signals that originate from the non-stem cells within the niche [1,39]. If holoclones are a more primitive progenitor cell then additional cycles of proliferation and differentiation will have to occur in order for the correct number

of niche to be established. However the mammosphere culture is purported to expand stem cell populations. This accounts for the differences in regeneration capacity in vitro and in vivo between COMMA-D holoclones and meroclones. Paraclones are comprised of differentiating cells therefore the majority of stem cells have been extinguished or differentiated to a more limited progenitor cell and do not form mammary outgrowths as efficient as meroclones upon transplantation. Holoclones gave limited mammary outgrowths when transplanted suggesting the need for intercellular signaling between stem and non-stem cells.

Both MMTV-neu holoclones and meroclones formed mammary tumors when transplanted indicating that lack of expression of CD29 does not inhibit tumorigenesis in this model. Additionally cells dissociated from both holoclone tumors and meroclone tumors were able to form all three clone types. This result suggests there is cellular

plasticity within the tumor cells. The triple negative human breast cancer cell line MDA-MB-231 forms all three clone phenotypes but only holoclones express CD133 [40]. CD133 has been used as a cancer stem cell marker in a variety of cancer types [41]. In the case of the triple negative cells the holoclones demonstrated a greater potential for tumor formation based on clone formation on soft agar, CD133 expression, and expression of MMP-2 and -9 [40]. However the triple negative clones were not transplanted, this may be the reason for the differences between the triple negative cancer cells and the erbB2 overexpressing cancer cells.

Since much time and effort recently has gone into identifying stem cells based on cell surface markers, we wanted to characterize stem and progenitor cells in heterogeneous cell lines based on their progenitor and differentiation potential. Neither normal or tumor cell paraclones have the capacity to expand in vivo. Normal meroclones have the greatest regenerative potential while both tumor cell holoclones and meroclones retain tumorigenic capacity. From these observations we conclude that the clone type that forms is predicated on the stage of differentiation of the cell from which it arose. Holoclones arise from early stage stem and progenitor cells, meroclones arise from later stage progenitor cells and paraclones arise from differentiating cells. Each clone type can be defined by specific characteristics including in vivo potential.

## CONCLUSIONS

Cell lines derived from normal mouse mammary epithelial cells and mouse mammary tumors have the capacity to form three different clone types. Each clonal subtype displays distinct marker patterns and repopulation capacity.

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