In vivo transplantation into the mammary fat pad represents the cornerstone assay for evaluating mammary stem cell (MaSC) activity. Pioneering work has shown that mammary epithelial outgrowths can be generated in de-epithelialized (or cleared) fat pads transplanted with explants or admixtures of mammary cells [1]. More recently, MaSCs have been prospectively isolated and demonstrated to exhibit multilineage differentiation and self-renewal properties through the transplantation of limiting numbers of empirically derived cell subpopulations. A MaSC-enriched basal population was identified on the basis of high expression of integrin β1 (CD29) or integrin α6 (CD49f) and moderate levels of CD24 [2,3], with an estimated stem cell frequency of 1 in 60. Using CD24 as a single marker, the CD24<sup>mod</sup> subset was shown to comprise almost all repopulating activity [4,5].

A number of recent studies have incorporated the reconstituted extracellular matrix Matrigel (BD Biosciences) in their mammary transplantation assays, with a view to creating an improved microenvironment for the implantation of stem cells. These studies have included the transplantation of unsorted mammary cells, in which as few as 100 cells could reconstitute an entire mammary gland [6], and the transplantation of sorted epithelial subpopulations embedded in Matrigel [7-10]. Interestingly, Matrigel was recently shown to enhance melanoma cell tumor-initiating capacity several-fold [11]. Given the increasing use of Matrigel in transplantation assays, we have directly assessed the effect of this matrix on the repopulating capacity of two distinct subpopulations isolated from normal mouse mammary glands: the MaSC-enriched subset and the luminal cell subset, the latter of which comprises committed luminal progenitor and mature luminal cells. We report here that the luminal subpopulation can yield limited ductal outgrowths, but only in the presence of Matrigel. These data raise the possibility that rare bipotent cells in this subset are activated by matrix components or that committed luminal progenitor cells can undergo dedifferentiation. In either case, these cells do not represent true MaSCs.

MaSCs have previously been shown to lie within the CD29<sup>hi</sup> (or CD49f<sup>hi</sup>) CD24<sup>+</sup> population, while extensive transplantation assays of luminal cell fractions including the CD61<sup>+</sup> luminal progenitor subset have demonstrated that this luminal population lacks repopulating potential [2,3,12]. In human breast tissue, stem cell activity was similarly demonstrated to occur in the basal population [13,14]. To address the influence of Matrigel on in vivo mammary repopulating capacity, we transplanted double-sorted cells from the MaSC-enriched subset (CD29<sup>hi</sup>CD24<sup>+</sup>) and the luminal subset (CD29<sup>hi</sup>CD24<sup>+</sup>) in either 0%, 25% or 50% Matrigel. Donor cells were derived from Rosa26 mice to allow definitive identification of outgrowths from implanted cells by virtue of β-galactosidase activity. Cells within the CD29<sup>hi</sup>CD24<sup>+</sup> subset were transplanted at limiting dilution, in which 1 in 75 cells is estimated to be a MaSC [2], while an excess of luminal cells (1,000 cells)
were injected. Matrigel at both concentrations was found to substantially enhance the mammary repopulating frequency of the MaSC-enriched subpopulation, with the percentage of outgrowths from transplanted cells almost doubling in the presence of 50% Matrigel compared with no Matrigel (Figure 1). In general, more extensive filling of the fat pad was apparent in the presence of this matrix. These data are compatible with the increased engraftment observed upon inclusion of 50% Matrigel [9]. Constituents within Matrigel may enhance the viability and/or activity of stem cells, resulting in increased repopulating capacity.

Unexpectedly, transplantation of the luminal subpopulation in Matrigel gave rise to small branched structures (Figure 1a,b): 10.7% and 22.5% were observed in the presence of 25% and 50% Matrigel, respectively. No outgrowths, however, were generated from this subpopulation in the absence of Matrigel, consistent with previous studies [2,3]. Notably, only diminutive outgrowths arose from luminal subset cells inoculated in 50% Matrigel, although each structure exhibited ductal branching from a central point and was therefore scored (Figure 1b,c). In the case of 25% Matrigel, the structures filled approximately 1% of the fat pad.

### Table 1

| Cell population | % Matrigel in transplant buffer |
|-----------------|--------------------------------|
|                 | 0     | 25    | 50    |
| CD29^hiCD24^-   | 18/40 | 17/28 | 35/40 (87.5%) |
| CD29^loCD24^-   | 0/40  | 3/28  | 9/40  (22.5%) |

Figure 1. Effect of Matrigel on the transplantation of mammary epithelial cell subpopulations. (a) Table showing the number of outgrowths per number of mammary fat pads injected with either 75 CD29^hiCD24^- (mammary stem cell (MaSC)-enriched) cells or 1,000 CD29^loCD24^- (luminal) cells, in either 0%, 25% or 50% Matrigel. Single cell suspensions were prepared from the mammary glands of 8-week-old to 10-week-old FVB/N-Rosa26 female mice, labeled with fluorochrome-conjugated antibodies and double-sorted as described [2]. The MaSC-enriched and luminal cell populations were identified following depletion of endothelial and hematopoietic cells using anti-CD45, anti-CD31 and anti-TER119 antibodies. Cells were injected (10 μl volume) into the cleared inguinal mammary fat pads of 3-week-old FVB/N female recipients and were collected 8 weeks post transplantation for X-gal staining. β-Gal^+ branched ductal structures were scored as positive. Data are shown for four independent experiments. (b) Images of X-gal-stained outgrowths: outgrowth derived from transplantation of 75 CD29^hiCD24^- cells in 50% Matrigel (top), and largest outgrowth obtained from transplantation of 1,000 CD29^loCD24^- cells in 50% Matrigel (bottom). Bar = 1 mm. (c) Bar chart representation of mammary outgrowths as a function of fat-pad filling following transplantation of each subpopulation. The axes shown differ for the two populations, since very few structures were generated by the CD29^loCD24^- population and these did not exceed 5%. Data are shown for four independent experiments. MFP, mammary fat pad.
Secondary transplantation experiments were carried out from luminal cell-derived (n = 3) or MaSC-derivative (n = 3) outgrowths to determine whether the luminal cell-derived outgrowths contained cells with self-renewal capacity. No outgrowths were present in 20 recipient glands, whereas prominent ductal outgrowths were evident in recipient glands from all three MaSC-derived outgrowths (15/20). Thus the Matrigel-associated luminal cell-derived (CD29<sup>hi</sup>CD24<sup>+</sup>) outgrowths did not exhibit self-renewal properties, a hallmark feature of stem cells.

Contamination of this luminal subpopulation (double-sorted and purity confirmed by reanalysis) with MaSCs seems unlikely as no outgrowths were evident in the absence of Matrigel, and no extensive outgrowths were ever observed. Rather, Matrigel may be providing a microenvironment that activates rare bipotent progenitor cells capable of regeneration, albeit limited. Alternatively, luminal progenitor cells within this subpopulation may occasionally adopt a more primitive state. These data differ from those recently reported in which Matrigel was found to be necessary for the generation of outgrowths from both the CD49<sup>hi</sup>CD24<sup>med</sup> and CD49<sup>hi</sup>CD24<sup>hi</sup> subpopulations [10]. Contrary to the findings described here, a similar degree of engraftment was noted for each population, perhaps reflecting the large number of cells transplanted (50,000 cells) [10].

It is conceivable that the activation of signaling pathways by Matrigel components can stimulate certain cells to acquire a more primitive state. Matrigel is a solubilized basement membrane extracted from Engelbreth–Holm–Swarm mouse sarcoma and is rich in laminin, collagen IV, proteoglycans as well as a number of different growth factors [15]. The nature of the substance or growth factors in Matrigel that may confer a more permissive environment for progenitor activity is yet to be determined. Growth factor-reduced Matrigel could be considered an alternative to complete Matrigel to perhaps distinguish effects of the substratum components from those of growth factors on mammary reconstitution.

Matrigel has been widely used to study tumor cell invasion, and an altered extracellular matrix has been shown to promote tumorigenesis [16]. In xenotransplantation assays to identify cancer stem cells in primary tumors, it is pertinent that only the cancer stem cell fraction and not the negative fraction had tumor-initiating capacity in mice when inoculated in Matrigel [17]. This reconstituted basement membrane, however, has been found to facilitate tumorigenesis of human breast cancers, squamous cell carcinomas and teratomas in mice [18-20], suggesting it has the potential to provide tumor cells with additional survival and/or proliferative signals. The influence of Matrigel on established tumors, however, is a distinct question from its impact on normal cells.

In summary, our data suggest that, in addition to increasing the rate of engraftment by MaSCs, Matrigel appears to promote progenitor activity in the luminal subset that is not seen in its absence. It is important to note that these cells with limited regenerative potential are distinct from bona fide MaSCs that lie within the basal population and should not be scored as such. A degree of caution should thus be applied to interpreting data from mammary cell transplantation experiments that incorporate Matrigel, particularly when transplanting high cell numbers. Additional studies (such as comparison of complete Matrigel and growth factor-reduced Matrigel) will be required to resolve the question of whether it is more or less physiological to include this matrix in transplantation assays for MaSC function.

Abbreviations
MaSC, mammary stem cell

Competing interests
The authors declare that they have no competing interests.

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