THE SEARCH FOR STEM CELLS IN HUMAN BREAST MILK

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Summary

Mammary stem cells (MaSC) are under stringent scrutiny these recent years, due in no small part to the fact that breast cancer is the most common cancer among females worldwide and that MaSC have been extensively studied as a system to delineate the pathogenesis and treatment of breast cancer. However, research on MaSC requires tissue biopsies which limit the quantity of samples available. Taking reference from other systems with epithelial cells' lining, studies have document the presence of the of the stem cell in the luminal discharges due to the proximity of the stem cell niche to their luminal cavities. For example, mesenchymal progenitor cells have been isolated through the collection of human menstrual blood and urothelial stem cells have been derived from urine. Extrapolating these reports to a tissue of common lineage, they suggest the plausibility of mammary stem/ progenitor cells being shed into human breast milk (HBM) either by means of sloughing or active shedding into the lumen for yet unknown purposes during a time of intense cellular turnover.

In my study, I hypothesised that stem cells are shed into the HBM and aimed to isolate them from HBM. Successful derivation of these cells from HBM may aid progress of research in mammary stem/ progenitor cells by providing a novel and non-invasive source. In addition to allowing a comprehensive understanding of the various components of HBM throughout the entire lactational period, this novel cell source may contribute to the reconstruction of the mammary tissues, and the unshedding of mechanisms behind the link between MaSC and breast cancer.

HBM contains a mixed population of cells of haemopoietic, mesenchymal and neuro-epithelial lineages. Further analysis of the adherent cultured cells reveals a

heterogeneous population of cells with differential expression of cytokeratin (CK)5, CK14, CK18 and CK19. In addition, there was a small population of nestin-positive cells (16.0±2.6%), of which 53.1±4.2% and 55.2±2.85% co-stained with CK5 and CK 19 respectively, and only 22.3±1.5% and 26.0±2.7% co-stained with the more mature epithelial markers, CK14 and CK18 respectively. This suggests a hierachical model of mammary cells within our culture system with the nestin+ cells being the putative MaSC followed by the intermediates of nestin+CK5+ and nestin+CK19+ cells, which are in turn more immature than the nestin+CK14+ and nestin+CK18+ cells. The terminally differentiated cells in our model would be the nestin-CK14+ and nestin-CK18+ cells.

In order to prospectively isolate putative MaSC for characterisation, two different approaches were undertaken. Firstly, flow cytometric sorting of side population (SP) cells revealed that 2% of cellular component of HBM were able to exclude Hoeschst 33342 dye, which selects for primitive stem cell populations. HBM SP cells co-expressed nestin but not the mature epithelial marker CK18. However, attempts to culture expand these putative MaSC in a wide range of in vitro conditions did not result in any mammary nor haemopoietic stem cells. Next, prospective isolation through selection of CD133 positive cells was done. Two percent of HBM were CD133-positive. These cells did not contain any haemopoietic activity, nor were attempts to expand them successful.

The derivation of MaSC from HBM would have availed a non-invasive source of stem cells of relevance to the understanding of lactation biology, oncogenesis and regenerative medicine. While some markers of primitive cell types of hierarchical

importance were detected, there was no evidence of any cell types with self-renewal and multi-lineage differentiation capacity. This may in part be due to poorly defined growth conditions, or the absence of such cell types in HBM.

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List of Abbreviations

ABCG2	ATP-binding Cassette G2
ACTH	Adenocorticotropic Hormone
ALP	Alkaline Phosphatase
BAS-CFC	Basophil Colony Forming Cell
bFGF	Basic Fibroblast Growth Factor
BFU-E	Burst Forming Unit-erythrocyte
BSA	Bovine Serum Albumin
CALLA	Common Acute Lymphoblastic Leukemia Antigen
CD	Cluster of Differentiation
CFC-Mix	Colony Forming Cells, Mixed
CFU	Colony Forming Units
CFU-E	Colony Forming Unit-erythrocyte
CFU-GEMM	Colony Forming Unit-granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM	Colony Forming Unit-granulocyte, macrophage
CFU-M	Colony Forming Unit- macrophage
CFU-S	Colony Forming Unit-spleen
CK	Cytokeratin
CNS	Central Nervous System
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMEM/ F-12	1:1 Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EOS-CFC	Eosinophil Colony Forming Cell
EPCAM	Epithelial Cell Adhesion Molecule
Epo	Erythropoietin
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FIL	Feedback Inhibitor of Lactation
FITC	Fluorescein Isothiocyanate
fMSC	Fetal Mesenchymal Stem Cell
GAPDH	D-glyceraldehyde-3-phosphate Dehydrogenase
GFAP	Glial Fibrillary Acidic Protein
GM-CFC	Granulocyte Macrophage Colony-Forming Cell
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
H&E	Hematoxylin and Eosin
HBM	Human Breast Milk
HBSS	Hanks Balanced Salt Solution
HDE	Hoechst Dye Exclusion
HSC	Haemopoietic Stem Cell
IFs	Intermediate Filaments
IGF	Insulin Growth Factor
IgG	Immunoglobulin G
IL	Interleukin
Lin	Lineage Cocktail 1

MaSC	Mammary Stem Cells
MEG-CFC	Megakaryocyte Colony Forming Cell
MMPs	Matrix Metalloproteinase
MNC	Mononuclear Cells
Msi	Musashi-1
MUC-1	Mucin-1
NES	Nestin
NFM	Neurofilament-M
NSP	Non-side Population
ON	Osteonectin
OP	Osteopontin
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween 20
PCR	Polymerase Chain Reaction
PDGFRα	Platelet-derived Growth Factor Receptor Alpha
PE	Phycoerythrin
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RPE	R-Phycoerythrin
RPMI	Roswell Park Memorial Institute Medium
Sca 1	Stem Cell Antigen 1
SCID	Severe Combined Immunodeficiency
SCF	Stem Cell Factor
SEM	Standard Error Mean
sIgA	Secretory Form of Immunoglobulin A
SMA	Smooth Muscle Actin
SP	Side Population
SVZ	Subventricular Zone
TEBs	Terminal End Buds
TGF-R	Transforming Growth Factor Receptor
TPO	Thrombopoietin
WCP	Whole Cell Population

1 Introduction

Lactogenesis and Lactation

1.1.1 Lactogenesis

Lactogenesis is defined as the process by which the mammary gland develops the capacity to secrete milk (Neville 1999). It can be divided into phase I and II. Phase I occurs during mid-pregnancy with the initiation of secretory differentiation with the synthesis of milk proteins and enzymes important to milk formation (Hartmann and Cregan 2001; Neville et al. 2001). While milk secretion through the ducts has not yet set in, increased concentration of lactose and α-lactalbumin can be detected in the plasma (Arthur et al. 1991; Atwood and Hartmann 1995) and lactose can also be detected in the urine (Atwood and Hartmann 1995). Phase II begins with the onset of copious milk secretion, typically within the first four days postpartum, following the drop in circulating progesterone levels (Atwood and Hartmann 1995; Neville 1999; Hartmann and Cregan 2001; Neville and Morton 2001).

1.1.1.1 Regulation of Lactogenesis

It has been well established that abrupt changes in the plasma concentrations of the hormones of pregnancy set lactogenesis in order. In a developed mammary epithelium, the constant presence of prolactin near 200ng/ mL and a fall in progesterone is essential for the onset of lactogenesis II (Kuhn 1983). In humans, this is illustrated when removal of placenta, the source of progesterone is necessary for initiation of milk secretion (Neifert et al. 1981) and that delayed lactogenesis occurs when placental fragments, capable of secreting progesterone is retained (Neifert et al. 1981). Besides the fall in progesterone, other hormones are required for the onset of lactogenesis II. Prolactin is essential for sustained lactation after the fall in placental lactogen that accompanies the

decline in progesterone levels post removal of placenta (Neville and Morton 2001). However, the amount of milk secreted is not directly related to the concentration of prolactin in the blood, but rather local mechanisms within the mammary gland (Neville 1999). One of them, the protein factor, FIL is secreted with the other milk components into the alveolar lumen and acts by reversible blockade of constitutive secretion in the mammary epithelial cell (Peaker and Wilde 1996). Thus, an increase in emptying brings about an increase in the rate of milk synthesis over a period of days and conversely, decreased emptying brings about a reduction in milk synthesis.

1.1.1.2 Changes during Lactogenesis

Milk production starts at less than 100mL/ day on day one postpartum and increases to an average of 500mL by day four. During this period, milk composition alters massively as lactogenesis progresses from Phase I to Phase II, with the fall in sodium and chloride concentrations and a rise of lactose concentration (Neville et al. 1991). Concurrently, the concentrations of secretory immunoglobulin A and lactoferrin increase dramatically and remain high up to 48hrs after birth (Lewis-Jones et al. 1985). Their concentrations fall rapidly after day two partly due to dilution as milk volume secretion increases, but their secretion rate is still substantial (2–3g/ day for each protein throughout lactation). Oligosaccharide concentrations are also high in early lactation, comprising as much as 20g/ kg of milk on day four (Coppa et al. 1993; Coppa et al. 1999), falling significantly to a level of 14g/ kg of milk on day 30. The substantial volume increase between 36hrs and 96hrs reflects a massive increase in the rates of synthesis and/ or secretion of almost all the components of mature milk (Neville et al. 1991), including but not limited to lactose, protein (primarily casein) (Patton et al. 1986; Chen et al. 1998), lipid, calcium, sodium, magnesium, and potassium.

1.1.1.3 Factors Associating with Lactogenesis

There are numerous factors potentially associated with lactogenesis in humans which can be summarised in the following table.

	Biological	Behavioural
Maternal	Parity	Motivation to breastfeed
	Mode of Delivery	Social support
	Labour Experience	Nursing frequency
	Body mass index	Use of supplements
	Smoking	Use of pacifiers
	Breast/ Nipple abnormality	Breastfeeding experience
	Breast/ Nipple surgery	
	Illness	
	Anxiety and stress	
	Retained placental fragments	
	Hypothyroidism, hypopituitarism	
	Ovarian theca-lutein cyst	
	Polycystic ovarian syndrome	
	Postpartum haemorrhage with Sheehan's syndrome	
	Hormonal contraceptive administration first week postpartum	
Infant	Birth weight	Temperament
	Gestational Age	Suckling style
	Suckling ability	

Table 1-1: **Factors associated with lactogenesis.** Adapted from (Dewey et al. 2001; Hurst 2007).

The problem with failed lactogenesis can be subdivided into preglandular, glandular and post glandular (Morton 1994). An example of preglandular would be hormonal causes, such as retained placenta or lack of pituitary prolactin. Glandular causes might be surgical procedures, such as reduction mammoplasty or, possibly, insufficient mammary tissue. Lastly, postglandular would be any cause for ineffective or infrequent milk removal. This latter aspect has received insufficient attention. Observational studies have suggested that milk removal and/ or effective suckling are necessary for milk volume increase, at least in a proportion of females (Aperia et al. 1979; Morton

1994), although this is in contrary with other studies. Kulski et al suggested that milk removal was not necessary (Kulski et al. 1978) and Chen et al showed that it was the time of the first feeding and the breastfeeding frequency on day 2 postpartum that was positively correlated with the milk volume (Chen et al. 1998).

Two other major risk factors have been shown to be responsible for delayed lactogenesis: long duration of labour (for natural deliveries) and urgent caesarean section, both of which are strongly related to the amount of stress experienced by both the mother and baby during parturition (Chen et al. 1998; Dewey 2001). These results affirm firstly, the impairing of milk ejection reflex by affecting oxytocin release during a feed and secondly, both maternal and fetal stress during pregnancy and childbirth are associated with impaired lactogenesis. Emotional stress postpartum is found to impair lactogenesis as well, highlighting the importance of additional care and guidance for women who experience highly-stressful circumstances (Dewey 2001). Initiation of lactogenesis has been found impaired with poorly-managed diabetes (Neville et al. 1988; Arthur et al. 1989; Neubauer et al. 1993) and high body mass (Hilson et al. 1997), which have more recently been found to be associated with delayed lactogenesis II (Chapman and Perez-Escamilla 1999; Chapman and Perez-Escamilla 2000).

Dissecting the mechanisms in which various factors influence lactation and breastfeeding is required as a basis for analysing the possible effects on this process in situations where delayed or failed lactogenesis is suspected. Recognizing when and how to intervene in order to properly detect and assess the degree to which lactation is compromised will allow for individualized interventions and appropriate follow-up which would be invaluable in managing the initiation of breastfeeding, especially in

mothers of sick infants as well as in sick mothers of well infants (Neville and Morton 2001; Hurst 2007). A collaborative effort between nurses, midwives, physicians, and lactation consultants will serve each mother with a coordinated and individualized plan of care for her unique situation. In addition, with the knowledge of the micro changes accompanying lactogenesis, as well as identifying compounds if any that may hinder milk secretion would provide a new index for predicting which women are likely to have problems initiating lactogenesis II (Neville and Morton 2001). On the whole, all these collectively would bring forward treatment and management soonest possible and on top of that, helping the mother recognize her full lactation potential, even when it falls short of exclusive breastfeeding, can result in a feeling of success and accomplishment (Hurst 2007).

1.1.2 Lactation

Lactation is the process of milk secretion which is prolonged as long as milk is removed from the gland on a regular basis. It is the defining characteristic of all animals of the class Mammalia, whereby there is the production of an externally secreted fluid, milk that is designed specifically to nourish the young of the species. In humans, breast milk has been recognized as the preferred nutrition for infants and exclusive breastfeeding up to six months. Thereafter, continued breastfeeding complemented by solid foods being recommended up to 2 years of age by international agencies and several US health organisations (World Health Organisation 1989; Institute of Medicine 1991; American Academy of Paediatrics 1997).

1.1.2.1 Milk Volume Production

Milk secretion is a robust process that proceeds under normal physiologic principles as previously outlined [in Section 1.1.1] in at least 85% of postpartum women. Initiated at less than 100mL per day, it increases 36hrs after delivery, and continues to increase dramatically for the next 48hrs before plateauing off around 4 days postpartum (Arthur et al. 1989; Neville 1999). A meta-analysis of the volume of milk secreted by exclusively breastfeeding women showed that milk volume is remarkably fairly constant at about 800 mL per day in different populations throughout the world (Neville 1999).

With assistance in the techniques of breastfeeding, anecdotal evidence suggests that at least 97% of women can successfully breastfeed their infants. The lack of success is largely due to the ease in substituting breast milk with formula when infants have yet to adapt to the breast, at least in the Western countries (Neville 1999). However, in recent years, the increasing awareness of the benefits of breast milk is causing a paradigm shift in terms of proportion of women choosing to breastfeed who are further encouraged by the increasing number of support groups worldwide.

1.1.2.2 Effects of Lactation

1.1.2.2.1 Effects on breastfeeding female

In humans, the metabolic demands of breastfeeding increase the maternal metabolism by about 20% of the usual metabolic output of a moderately active woman (Prentice and Whitehead 1987). Hence, very little adjustment, for instance, a slight increase in food intake or a slight increase in weight loss, is needed to compensate for the increase

in metabolic needs for secretion of breast milk (Neville 1999). While the adjustment in food intake is not drastic, calcium loss from their bones by the postpartum female would be significant (Cross et al. 1995; Krebs et al. 1997), due to the lack of oestrogen during the period of postpartum infertility when menses have yet to be resumed and therefore, food intake has to be adjusted to ensure increased calcium intake.

It has been suggested that breastfeeding reduces stress levels in lactating females. Using rodents, it was found that a reduction in the usual endocrine response to stress including adrenocorticotropic hormone (ACTH), corticosterone, catecholamines, oxytocin and prolactin was found to be associated with lactation (Neville 1999). This result was similar in lactating women when their plasma levels of ACTH, cortisol and adrenaline were compared against a matched group of nonlactating women after graded treadmill exercise to simulate stress (Altemus et al. 1995).

There have also been several studies highlighting the protective role of breastfeeding with regards to breast (Byers et al. 1985; McTiernan and Thomas 1986; Yuan et al. 1988; Layde et al. 1989; Yoo et al. 1992; Yang et al. 1993; Enger et al. 1997; McCredie et al. 1998) and ovarian cancers (Risch et al. 1983; Rosenblatt and Thomas 1993; Shoham 1994). With regards to breast cancer, it was found that the level of relative risk reported varied from approximately 0.54 to 0.85 for the first three to six months of breastfeeding, from 0.39 to 0.71 at twelve months of breastfeeding, 0.4 to 0.72 for more than two years, and 0.35 for more than six years (Labbok 2001). The biological reasoning for this association include differences in cellular milieu (Kennedy 1994) and the lack of maturation of the mammary gland. Back in 1983, Risch et al found a protective effect of lactation (relative risk of 0.79 per year of lactation) in a

retrospective study of newly diagnosed patients with epithelial ovarian cancer although it was later proved to be accounted by the inhibition of ovulation through high prolactin levels generated by active breastfeeding (Risch et al. 1983). Subsequently by 1994, there were various conflicting findings on the protective effect of breastfeeding with ovarian cancer, with a balance towards a protective effect of breastfeeding in reducing risk of ovarian cancer (Shoham 1994).

One of the most marked effects of lactation is its effect on fertility. Due to the presence of the suckling stimuli, luteinising hormone secretion remains in its suppressed state and secretion of ovarian steroids remains low. This results in natural contraception in the form of low fertility postpartum, in the presence of active breastfeeding (Neville 1999). This postpartum suppression of fertility is believed to play an important role in birth spacing on a population basis in developing countries where prolonged breastfeeding is the norm and the use of supplementary feeding is delayed (Neville 1999).

1.1.2.2.2 Effects on infant

There have been several studies reporting on the benefits of breast milk for the infant. These studies have reported a benefit of breastfeeding with respect to the reduction in the incidence and severity of infantile diarrhoea, respiratory and urinary tract infection, otitis media, Haemophilius influenzae meningitis, and other infections as well as the rate of sudden infant death syndrome (Pisacane et al. 1992; Owen et al. 1993; Baker et al. 1998; Wilson et al. 1998). Specifically, Baker and his colleagues found through analysing data from a survey of 8,501 women that breastfeeding for three or more months reduced the prevalence of wheeze and diarrhoea in the first six months of life

(Baker et al. 1998). Longitudinal studies also found that the protective effect of breast milk is dose dependent (Duncan et al. 1993; Duffy et al. 1997; Scariati et al. 1997; Cushing et al. 1998).

It is becoming evident that breastfeeding also protects infants against illness beyond weaning, as indicated by several studies that included the follow-up of infants beyond the first few months of life (Davis et al. 1988; Koletzko et al. 1989; Takala et al. 1989; Howie et al. 1990; Gerstein 1994; Dewey et al. 1995; Saarinen and Kajosaari 1995; Wright et al. 1995; Wilson et al. 1998). Long term benefits of breastfeeding have been associated with reduced risks of developing allergic diseases, Type I diabetes mellitus, Crohn's disease and malignant lymphoma (Davis et al. 1988; Koletzko et al. 1989; Gerstein 1994; Saarinen and Kajosaari 1995). Breastfed infants are also believed to be at an advantage with respect to their long-term cognitive development and lower rates of neurologic disabilities (Lucas et al. 1992; Rogan and Gladen 1993; Lanting et al. 1994; Temboury et al. 1994).

In addition to its nutritional, anti-infective, immunologic, and developmental effects, breastfeeding is unique for its mode of feeding with important advantages of hygiene particularly in developing countries as well as physical and emotional bonding of mother and child (Kunz et al. 1999; Rodriguez-Palmero et al. 1999).

1.2 Breast Biology

1.2.1 Anatomy

Mammary tissue can be subdivided into the parenchymal and the stromal components. The parenchymal component is formed by a number of 'treelike' glandular structures derived from dichotomous branching of each of several ducts arising from the nipple. The major functional units of these glands are the lobular structures, situated at the end of the terminal ductules, which comprise several smaller blind ending ductules often referred to as terminal ductal lobulo-alveolar units (TDLUs), which has been defined by Russo and Russo as a cluster of approximately 11 small ductules or alveolar buds around a terminal duct that is embedded in specialized intralobular stroma (Russo and Russo 1987). These TDLUs are lined by a continuous layer of luminal epithelial cells, which in turn are enmeshed by myoepithelial cells that contact the basement membrane. This entire structure is then surrounded by delimiting fibroblasts, and embedded in a specialized intralobular stroma (Howard and Gusterson 2000). These together with the adipose tissue and skin in the anatomical area constitute the stromal region (Mepham, 1983).

During pregnancy, the terminal end ducts undergo further differentiation with the MaSC differentiating into alveolar epithelial cells which are arranged into single-layer spherical structures known as alveoli, ductal epithelial cells lining the ducts, as shown in Fig. 1.1 and myoepithelial cells that surrounds the alveoli, forming a layer separating the ductal and alveolar epithelial cells from the basement membrane. Each of the alveoli has a central lumen in which the synthesised milk is stored till time of release.

Cells in the mammary gland lie in a collagenous connective tissue framework known as the extracellular matrix (ECM). Extracellular matrix of the mammary gland is a highly complex mixture containing collagen, fibronectin, laminin, glycosaminoglycans and others (Streuli 1993). The basement membrane of the secretory cells is also part of the ECM and is produced at least in part by the secretory cell itself.

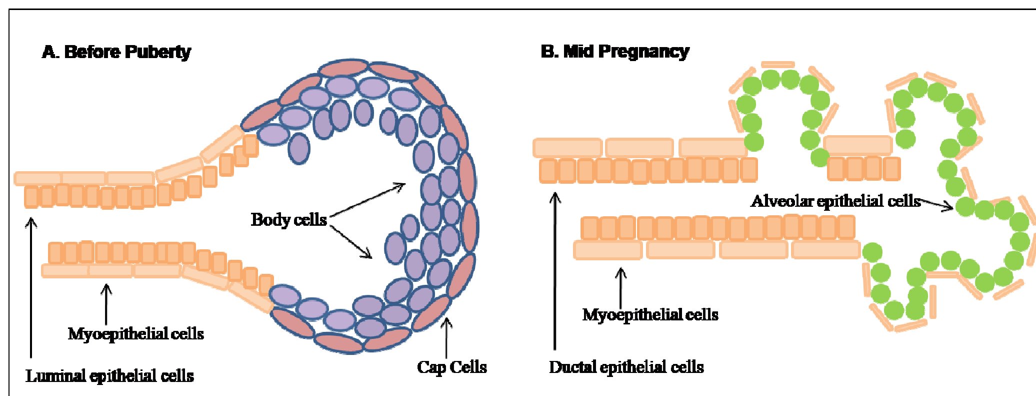


Figure 1-1 Schematic view of lobulo-alveolar clusters. Before puberty, the cells are arranged into terminal end ducts. Cap cells surrounding them are postulated to be multipotent stem cells (A). The terminal end ducts undergo differentiation during pregnancy forming alveoli. Alveolar and ductal epithelial cells that line the ducts of the clusters are surrounded by a layer of overlapping myoepithelial cells (B). *(Adapted from (Woodward et al. 2005).*

1.2.2 Cellular Component of Mammary Gland

1.2.2.1 Epithelial Cells

Mammary ducts are bilayered tubes composed of inner luminal epithelial cells surrounded by myoepithelial cells, which are in turn surrounded by an extracellular basement membrane (Anderson and Clarke 2004).

Luminal epithelial cells express the sialomucin MUC1, which is present on their apical membranes, whereas myoepithelial cells express the common acute lymphoblastic

leukaemia antigen (CALLA) and smooth muscle actin (Gusterson et al. 1986; Taylor-Papadimitriou et al. 1986; Taylor-Papadimitriou et al. 1992). In addition, each of these cell types also has a particular cytokeratin (CK) profile. Luminal epithelial cells express CK8 and CK18 while myoepithelial cells express CK14 (Taylor-Papadimitriou et al. 1989). Luminal epithelial cells synthesise the various milk components and empty them into the lumen where they are stored. These cells are cuboidal with their apical surface thickly covered with microvilli about 0.1µm in diameter and up to 0.5µm in length (Pitelka et al. 1983). Myoepithelial cells surround the alveoli and ducts (Figure 1-1). These contractile cells are more elongated than secretory cells and they contract in response to oxytocin, releasing milk from the lumen of the alveoli (Linzell 1952). In addition to fostering oxytocin-induced milk ejection by virtue of their contractile activity, myoepithelial cells are the cells that actually contact the basement membrane directly and are required for the production of ECM, including laminins. Thus they are ideally situated to transmit structural morphogenetic information from the basement membrane to the luminal epithelia. Indeed, isolated luminal epithelial cells (which do not form their own basement membranes) fail to form properly polarized hollow spheres when cultured in type I collagen gels and instead form solid lumen-less structures with reverse polarity unless myoepithelial cells are also added, in which case they do form aptly polarized, hollow, bilayered acinar-like structures (Gudjonsson et al. 2002a).

Apart from the two major epithelial cell types in the mammary gland, there also exists a third and less common intermediate population, the MaSC (Anderson and Clarke 2004). Mammary tissue has to be equipped with a ready source of MaSC to replenish the mammary gland through cycles of pregnancy, lactation and involution. This idea was

first suggested nearly three decades ago by the ability of a clonal murine epithelial cell line to differentiate into two different cell types (Bennett et al. 1978). Since then, the presence of mammary stem/ progenitor cells in the mammary gland has been established (Smith 1996; Gudjonsson et al. 2002b). The stem cell compartment within the breast was demonstrated to be localised within the luminal epithelial compartment (Pechoux et al. 1999; Gudjonsson et al. 2002a), a result which is consistent with an earlier finding that proliferating cells are found in the luminal population as cell division and expression of antigens associated with proliferation being exceedingly rare in the myoepithelial cell type (Joshi et al. 1986). These cells are responsible for the multiple cycles of proliferation and involution of the mammary tissue when necessary and will be further discussed in detail in Section 1.4.1.

1.2.2.2 Non-epithelial Cells

Apart from the epithelial cells which are responsible for the production and secretion of milk, capillary endothelial cells are also abundant in the highly vascularised mammary tissue. An extensive lymphatic system with lymphocytes and monocytes being common infiltrators is also present. The mammary tissue contains a large component of adipocytes in the stroma within populations of fibroblasts, which have been demonstrated in the pregnant murine models to transdifferentiate into secretory epithelial cells (Morroni et al. 2004).

1.2.3 Mammary Development

1.2.3.1 The Stages of Development

The mammary gland is one of the few tissues that undergo repeated cycles of development and regression in the adult animal. Development of the mammary gland is a highly dynamic and orchestrated process that occurs throughout postnatal life. Complete differentiation and maturation of each mammary compartment is a gradual process and has considerable variations between different individual woman, between each breast and even within each breast. Full differentiation of the mammary gland is only achieved in parous women (Hovey et al. 2002).

The process of breast formation is initiated during embryogenesis, with the formation of the milk streak at week 4 post fertilisation, which progresses into a bilateral mammary ridge (or milk line) during weeks 5 and 6 and then followed by the appearance of distinct placodes. Between 7th and 8th weeks of gestation, the formation and ingrowth of mammary bulbs begins, with further inward growth of the mammary parenchyma commencing at the 9th week. Between the 10th and 12th weeks, initial budding of the nascent mammary gland will be observed followed by the indentation resulting in formation of epithelial buds with notches at the epithelial-stroma border (Howard and Gusterson 2000; Hovey et al. 2002). A rudimentary ductal tree then forms by the branching of the parenchyma during the 13th-20th week which results in 15-25 epithelial strips or solid cords that eventually give rise to multiple galactophores at each nipple. During the latter stages of fetal development via the branching process and up to 32 weeks, the solid cords become canalized by apoptosis of the central epithelial cells. Finally, development of end vesicles comprising a monolayer of epithelial cells occurs in association with restrained lobulo-alveolar development between the 32nd and 40th

week of pregnancy (Hovey et al. 2002). Post-parturition, the infant breast undergoes menopausal-like involution whereby the ductal structures persist in a relatively quiescent manner until puberty, beyond which breast development in males and females diverge.

At the onset of puberty, the female human breast undergoes variable amounts of terminal end buds (TEB) formation, duct elongation, dichotomous and lateral branching, terminal duct lobular unit formation and stromal expansion directed by concurrent modifications in hormones and growth factors across the various reproductive stages (Hovey and Trott 2004; Sternlicht 2006). The male breast on the other end, remains quiescent but capable of further development under certain circumstances from exogenous estrogens, liver failure and stimulation from drugs, resulting in gynecomastia (Sternlicht 2006).

Further into the female's lifespan, at the onset of pregnancy and associated changes in hormonal and local environment, alveolar development progresses with the mammary epithelial cells within the gland attaining their unique ability to synthesise various milk components. At the last stages of gestation, the distal portion of the mammary ducts develops into alveolar structures and mammary epithelial cells appear secretory, such that with parturition, functional lactogenesis can take place (Hovey et al. 2002; Hovey and Trott 2004). This process is driven by the systemic hormonal stimuli that elicit local paracrine interactions between the developing epithelial ducts and their adjacent mesenchyme, to be further discussed in Section 1.2.3.2 (Sternlicht 2006).

Involution occurs when regular extraction of milk from the gland ceases. Milk stasis is the key signal for the alveolar secretory epithelial cell to undergo apoptosis and be removed by phagocytosis. The alveoli will collapse and by day 6, disintegrated completely, with both the stromal and epithelial components remodelled, while the ducts remain intact (Richert et al. 2000). This involves an orderly sequence of events including cessation of milk secretion, increased secretion of lactoferrin (Hartmann 1973), opening of the tight junctions, apoptosis of the mammary epithelium (Strange et al. 1992), and changes in secretion of proteases (Lund et al. 1996), followed by the remodelling of the ECM, after which the gland returns nearly to its prepregnant state, after 3 weeks (Neville 1999; Anderson and Clarke 2004). However, it appears that the human mammary gland does not revert to the virgin state but to a more differentiated form in terms of number of lobules in each TDLU (Russo and Russo 1987). It is believed that the reduction in breast cancer risk afforded by an early first full-term pregnancy may be related to the fact that the gland is left in a more differentiated state following involution (Russo et al. 2001).

Eventually, the mammary gland undergoes another round of involution at menopause (Anderson and Clarke 2004). For this phase of involution, there is regression of ducts and lobules, and adipose tissue replaces the glandular epithelium and interlobular stroma with the eventual result of sparse scattering of atrophic acini and ducts through the tissue (Howard and Gusterson 2000).

1.2.3.2 Regulators of Development

Each stage of the differentiation process is tightly controlled by both soluble factors such as hormones and growth factors and the interactions of cells with the environment (Guyette et al. 1979; Topper and Freeman 1980; Hobbs et al. 1982).

Briefly, the ductal tubes are compactly surrounded by the myoepithelial layer, preventing the direct interaction of the ductal epithelium with the basement membrane in the nulliparous female (Figure 1-1). Increased levels of progestins, oestrogens and placental lactogen during pregnancy allow the budding of the alveolar epithelium. This brings most alveolar epithelial cells in close proximity to the basement membrane as myoepithelial cells build a loose network that wraps the alveolar structures. Involution of the mammary gland occurs when lactation ceases. The alveolar structure starts to disintegrate and the basement membrane is removed by ECM-degrading metalloproteinases (Knight 1995).

1.2.3.2.1 Hormonal control

For the hormonal influence, several studies have shown that oestrogen, progesterone, prolactin (PRL), growth hormone (GH), and thyroid hormones are essential for ductal elongation, branching, and alveolar budding, specifically, oestrogen and its corresponding receptor required for adolescent branching while progesterone and its corresponding receptor is required for adult tertiary side-branching (Sternlicht 2006). Adrenal steroids, prolactin (PRL), growth hormones (GH), thyroid hormones, oxytocin, and insulin are required for complete lobuloalveolar development and milk synthesis, secretion, and lactation. Some of these hormones (oestrogen, progesterone, PRL, and

GH) appear to be inductive while others play a more permissive role (Hovey et al. 2002).

1.2.3.2.2 Interaction with environment

Apart from hormonal control, it has also been established that there are several other factors involved in branching morphogenesis, such as epidermal growth factor (EGF) signalling through its ligand (Coleman et al. 1988; Kenney et al. 2003) and autocrine signalling through ECM-mediated regulation (Fata et al. 2004). Numerous culture-based studies show that, in addition to providing a structural foundation for cells, ECM components convey contextual information through cellular adhesion molecules, such as integrins, that transmit external ECM-derived signals to the cell interior. Indeed, the three-dimensional ECM environment has been shown to affect virtually all aspects of cell behaviour, including cell shape, proliferation, survival, migration, differentiation, polarity, organization, and branching. In addition to their direct effects, various ECM components bind and sequester other signalling molecules that affect branching, such as amphiregulin, FGFs, Wnts, TGF-R, and IGF-binding proteins 1 to 6. Thus enzyme-mediated ECM remodelling can remove existing ECM signals, reveal hidden structural information, and release otherwise sequestered signalling molecules. Indeed, ECM degrading matrix metalloproteinase (MMPs) appear to have a path-clearing role in branching morphogenesis as well as an indirect cell signalling role that may reflect their ability to alter existing ECM signals, generate bioactive ECM fragments (for example cryptic integrin-binding sites on fibrillar collagen and a laminin-5 fragment that elicits epithelial cell motility), cleave cell–cell adhesion proteins (for example E-cadherin), degrade cell surface receptors (for example FGFR1), release ECM-bound growth

factors, inactivate IGF-binding proteins, activate latent TGF-1, and recruit other cell types to the surrounding stroma (Sternlicht and Werb 2001; Wiseman et al. 2003).

The importance of interaction of cells with the environment is further affirmed that tissue transplantation studies in which mammary epithelium and salivary mesenchyme (Sakakura et al. 1976) or skin epithelium and mammary mesenchyme (Cunha et al. 1995) were recombined demonstrate that mesenchymal cues control the branching pattern of the epithelium, regardless of epithelial origin.

1.3 Composition of Breast Milk

Human breast milk (HBM) has been humankind's first food for as long as the human race has existed. It is a complex biological fluid and contains many different constituents, which provide nutrients and also protection of the newborn against diseases before the immune system of the newborn is established. Being extremely dynamic in nature, HBM varies with increasing time after the birthing process, during each nursing feed, and with the mother's diet and certain diseases, adjusting to match the changing needs of the developing infant (Kunz et al. 1999). It provides a balanced nutrient composition as well as a number of conditionally essential nutrients and at least 45 different types and classes of bioactive factors, such as enzymes, hormones, and growth factors. Most of them play a role in supporting infantile growth and development (Bates and Prentice 1994; Koldovsky 1995). All contituents of HBM will be briefly discussed in this section.

1.3.1 Carbohydrates

Lactose is the major carbohydrate in HBM. The most important role of lactose is to regulate the water content in milk since its synthesis brings in a flow of water that dilutes other milk constituents like protein and fat (Davies 1983). It has been suggested that lactose aids the growth and development of the brain as one of its subunits, galactose is known to form a large part of the brain matter (Davies et al., 1983). Lactose also forms weak complexes with metal ions such as Ca^{2+} and Fe^{3+}, facilitating intestinal absorption of them by the infant (Davies 1983).

Human milk oligosaccharides may act as soluble receptors for different pathogens, thus increasing the resistance of breastfed infants to these potential pathogens (Kunz et al. 1999). The complex mixture of oligosaccharides that is present only in minute amounts among other uses, act as inhibitors of bacterial adhesion to epithelial surfaces, playing an important role in preventing infectious diseases in the newborn infant. They also seem to promote the development of a bifidus flora (Kunz et al. 1999), which can inhibit the growth of Escherichia coli and consequently protect breast-fed infants against gastrointestinal disease (Azuma et al. 1984).

HBM is also reported to contain glucose, galactose and fructose, which are possibly present as residuals of metabolic processes (Sheibak et al. 1978).

1.3.2 Lipids

Mammary alveolar cells synthesise milk fat from circulating lipids derived from the maternal diet as well as from maternal body stores. In addition, part of the milk fat can be synthesized *de novo* in the mammary gland from glucose (Thompson and Smith

1985). Synthesis of milk fat is stimulated by emptying of the breast through nursing and by prolactin secreted from the anterior lobe of the pituitary gland. The alveolar cells package and secrete the lipids into the lumen in the form of milk fat globuli. These have a hydrophobic core consisting of triglycerides, cholesteryl esters, and retinyl esters and are covered with bipolar or amphipathic compounds including phospholipids, proteins, cholesterol and enzymes in a loose network termed the milk fat globule membrane (Koletzko and Braun 1991).

The level of fat in HBM has been reported to vary from 0.4% to 10% rendering it the most variable of all milk components. 98% of lipids in human milk fat are triacylglycerols, followed by phospholipids (0.7%), cholesterol (0.5%), free fatty acid 0.08%, monoacylglycerols (trace) and cholesterol esters (trace) (Bracco et al. 1972; Bitman et al. 1983). The level of fat in HBM is also influenced by stage of lactation, time of milk sampling, frequency of milk output, stress and level of mammary gland stimulation prior to feeding (Packard 1982). Interestingly, studies have shown that there are no major differences in lipid composition in milk from term and preterm mothers, although there were more medium- and intermediate-chain fatty chains (10:0 compared to 14:0) in preterm than term milk (Bitman et al. 1983; Genzel-Boroviczeny et al. 1997).

The products of lipolysis and their monoglycerides after the digestion of triglycerides are potent microbicides that assist in controlling of infections in the stomach and small intestine (Hernell et al. 1989). Phospholipids in HBM, can be subdivided into major classes: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin and these phospholipids, being bipolar, act as emulsifiers to help maintain the globule emulsion (Jensen 1996). Lastly, the several

classes of sphingolipids and glycolipids, which are collectively known as gangliosides contribute to the host defense system in human milk by binding to cholera and other toxins (Laegreid et al. 1986).

1.3.3 Proteins

Human milk proteins are a source of peptides, amino acids and nitrogen for the infant, but also in the protein fraction reside other properties of HBM that further benefit the developing infant.

Protein in HBM comprises 40% casein and 60% whey proteins. Caseins associate under ionic conditions of milk to form micelles and these micelles are a source of amino acids for the infant. They also enable calcium and phosphorus to be transported in a stable form in milk (Davies 1983). Whey comprises a variety of proteins that supplement the immune and digestive system of the newborn and which will be discussed in the following sections.

1.3.3.1 Immunoglobulins

The main immunoglobulin in human milk is immunoglobulin A (IgA), although significant levels of lgG, IgM and IgD have also been detected (Goldman and Goldblum 1989). IgA exists in the secretory form (sIgA) in HBM and protects the infant against a range of enteric and respiratory pathogens; blocking the adhesion of potential pathogens to mucosal surfaces, hence preventing their colonisation and neutralising the toxins from microorganism (Telemo and Hanson 1996). It also prevents translocation of gut bacteria through the epithelial barrier, causes the agglutination of bacteria, and possibly offers protection against sepsis and function as natural

immunising agents that sensitises the infant to foreign antigens including microbial agents (Maxson et al. 1995; Steinwender et al. 1996; Garofalo and Goldman 1999); (Wold et al. 1994). As the sIgA molecule is too large to be filtered by the kidneys, the presence of a significantly higher output of intact IgA in urine of breastfed infants suggest that HBM can stimulate the production of IgA in the recipient infant (Goldblum et al. 1989; Prentice 1996).

1.3.3.2 Lactoferrin

The amount of lactoferrin in HBM declines throughout lactation, from 5-6mg/ mL in colostrum to 1.5mg/ mL in mature milk, defined to be milk produced beyond 10 days post delivery. Almost all lactoferrin in HBM is in the form of apo-lactoferrin, and it is this iron-free form that competes with siderophilic bacteria for ferric iron, disrupting their proliferation and preventing infection by these iron-requiring organisms (Brock 1980; Stuart et al. 1984). More recently, it was found that lactoferrin causes the release of lipopolysaccharide molecules from bacteria cell wall and sensitises the microorganisms to antibiotic and attachment by lysozyme (Packard 1982; Ellison and Giehl 1991). It also inhibits the complement system (Morgan et al. 1975), suppresses cytokine release from macrophages stimulated with bacterial products (Mattsby-Baltzer et al. 1996), suppresses in vitro antibody production (Duncan and McArthur 1981) and T lymphocytes' proliferative response to alloantigens and phytohemagglutinin (Richie et al. 1987). It has also been shown to bind to specific DNA consensus sequences that can confer lactoferrin-induced gene transcription (He and Furmanski 1995), and has been suggested to enhance iron absorption (Cox et al. 1979).

1.3.3.3 Prolactin

Prolactin augments the development of B and T cells (Gala 1991) and modulates differentiation and function of gut-associated lymphoid tissue, such as intraepithelial lymphocytes that express more prolactin receptors than neonatal splenocytes (Ellis et al. 1997). In addition to being a mitogen for T lymphocytes in culture (by induction of interleukin-2 and interleukin-2 receptors (Ellis and Picciano 1992; Grosvenor et al. 1993), milk-borne prolactin is essential for neuroendocrine development during a critical period of 2-5 days after birth in rats (Grosvenor et al. 1993).

1.3.3.4 Other Whey Proteins

A host of nucleotides are also present in HBM. Using animal models, it has been shown that nucleotides enhance mucosal development by stimulating growth and maturation of the gastrointestinal tract and intestinal repair after diarrhoea (Uauy et al. 1990; Bueno et al. 1994). The immunomodulatory activity of nucleotides has been associated with the enhancement of T-cell maturation and function (Van Buren et al. 1985), improvement of delayed cutaneous hypersensitivity and alloantigen-induced lymphoproliferation (Van Buren et al. 1983) and partial resistance to infection with bacteria or other pathogen (Kulkarni et al. 1986).

Some soluble cell adhesion molecules and their ligands detected in HBM aid in the defence against pathogens as well (Buescher and Malinowska 1996; Schwertmann et al. 1996).

Several cytokines, chemokines and colony-stimulating factors have been discovered in human milk. Interleukin 6 is believed to enhance IgA1 and IgA2 production (Fujihashi

et al. 1991) by the intestinal and bronchial epithelial cells which expressed the appropriate receptor (Shirota et al. 1990; Takizawa et al. 1996). Interleukin 10 exerts anti-inflammatory effect in the form of suppressing the functions of macrophage, T-cell and natural killer cells' function by blocking inflammatory cytokine synthesis and several accessory cell functions (Kuhn et al. 1993; Moore et al. 1993; Garofalo et al. 1995). It also induces activated B cells to produce large amounts of IgM, IgG and IgA (Rousset et al. 1992). Together with interferon-γ, they modulate in vitro epithelial integrity and ion transport along the stomach wall of the infant, suggesting the expression of functional receptors for these cytokines by human epithelial cells (Archer and Johnson 1978; Adams et al. 1993; Madsen et al. 1997). Among the chemokines present, interleukin 8, growth-related peptide-α and RANTES were found to be the most potent chemotactic factors for intestinal intraepithelial lymphocytes (Ebert 1995).

There are also proteins in the form of enzymes and growth factors in HBM. Alpha-lactalbumin, alpha-amylase, bile salt stimulated lipase, α1-antitrypsin and antichymotrypsin aid digestion in the infant (Ebner and Schanbacher 1974; Lindberg et al. 1982; Packard 1982) with other functional enzymes like antiproteases, sulfhydryl oxidase, gamma-glutamyltransferase and xanthine oxidase whose functions are described briefly in Table 1-2. The large array of growth factors present in HBM are also tabulated and categorised in Table 1-3.

Enzyme	Function	Reference
Antiproteases	Protect bioactive proteins (enzymes, immunoglobulins) from hydrolysis in milk and in intestine of newborn	(Hamosh 1995)
Sulfhydryl Oxidase	Maintain structural and functional integrity of milk proteins, enzymes and immunoglobulins	(Isaacs et al. 1984)
γ-glutamyltransferase	Involved in endo and/ or exocytotic transport of proteins	(Binkley et al. 1975)
Xanthine Oxidase	Aid in secretion of milk fat droplets by peroxidising lipids	(Mather and Keenan 1983)

Table 1-2: **Functional proteins present in low levels in HBM.** Edited from (Packard 1982)

Constituent/ Activity
A. Hormones and other substances with growth-promoting potential assayed in milk
1. Epidermal growth factor
2. Insulin
3. Thyroxine
4. Cortisol
5. Luteinizing hormone-releasing hormone
6. Prostanglandins
7. Transforming growth factors
B. Demonstrated in vitro effect on growth of cells or tissues
1. Epidermal growth factor
2. Prostanglandins
3. Transforming growth factors
4. Lactoferrin
5. Granulocyte-colony stimulating factor
6. Macrophage-colony stimulating factor
7. Granulocyte-macrophage-colony stimulating factor
8. *Bifidobacterium bifidum* growth factors A and B

Table 1-3: **Growth factors present in HBM.**
Edited from (Morriss 1986) and (Garofalo and Goldman 1998)

1.3.4 Vitamins

The vitamin content of HBM is variable and their level depends on the interrelation of several factors like diet, stage of lactation, alcohol or drug use of which, the general health of the mother, particularly her diet is perhaps the most significant and pervasive factor. Vitamin A, E and K are present in HBM in significant amounts to exert their respective functional roles which range from aiding the infant's growth and development (Sommer et al. 1986; De Sole et al. 1987) to protecting against free radicals (Tappel 1965; Lucy 1972; Rodriguez-Palmero et al. 1999) and contributing to the coagulation process by supporting the synthesis of the relevant proteins (Rodriguez-Palmero et al. 1999).

1.3.5 Minerals

Sodium, potassium and chloride are the prevalent monovalent ions in HBM. The concentrations of these monovalent ions in HBM regulate in part, the opening of the tight junctions between epithelial cells (Atkinson et al. 1995). Calcium is essential for the development of bones, muscle contraction, the transmission of nerve impulses and clotting of blood (Packard 1982). Trace minerals in HBM include cobalt, manganese, molybdenum, aluminium, barium, chromium, nickel, copper, iodine, boron, fluorine, zinc, bromine, iron, selenium, strontium and rubidium. The major factor affecting concentration of minerals is advancing duration of lactation, which is associated with a decrease in sodium and an increase in potassium. Chloride drops over time, whereas calcium, free phosphate and magnesium increase (Rodriguez-Palmero et al. 1999). Maternal age, parity and lactation history also account for the variability of trace element concentrations (Howell et al. 1986).

1.3.6 Cells

The study of cells in human milk, first mentioned in 1844, has since then been studied extensively (Hamosh 2001). Epithelial cells, colostral cells, polymorphonuclear leukocytes, mononuclear phagocytes and lymphocytes have been reported in milk (Smith and Goldman 1968; Crago et al. 1979). The cell concentration and predominant cell type vary with duration of lactation and presence of breast inflammation.

1.3.6.1 Immune Cells

Colostrum defined as milk from day 1 to day 4 postpartum, contains 10^5 to $5x10^6$ leukocytes per mL of milk (Buescher and Pickering 1986). This concentration drops to the range of 10^5 per mL within the next six days of breastfeeding. The types of leukocytes found in HBM include macrophages, lymphocytes and polymorphonuclear leukocytes, with neutrophils and macrophages accounting for 90% of leukocytes in HBM (Xanthou et al. 1995). The neutrophils and macrophages present appear to be activated and were able to phagocytose and kill bacteria and fungi and they were also able to elicit antibody-dependent activity (Xanthou 1997). These cells also synthesise complements, lysozyme and lactoferrin, which acts synergistically to the cells.

Lymphocytes, though present at a much lower proportion, are also consistently found in HBM with T cells accounting for 80% of the total number of lymphocytes (Xanthou et al. 1995). Present in the activated form (Wirt et al. 1992), they were found to be less efficient in their cytotoxic capability when compared against the peripheral cells of the same individuals (Nair et al. 1985) but capable of generating certain lymphokines and growth factors (Skansen-Saphir et al. 1993). B lymphocyte deficient mice were able to

avoid severe infections when breastfed by normal mouse (Arvola et al. 2000) thus suggesting that maternal lymphocytes compensate for the developmental delay of the infant (Goldman et al. 1998). Maternal lymphocytes in HBM have been shown to enter the circulation of the offsprings, presenting themselves in the intestinal mucosa, lymph nodes and ducts (Weiler et al. 1983; Slade and Schwartz 1987; Jain et al. 1989; Arvola et al. 2000). This suggests that lymphocytes from milk may be able to induce not only local and also a systemic immune response (Xanthou 1997). Phagocytes count falls after 4 weeks of lactation, beyond which 90% of the cellular content in breast milk are epithelial cells (Brooker 1980).

1.3.6.2 Epithelial Cells

Three types of epithelial cells have been recognised in HBM, namely the secretory, myoepithelial and ductal cells. They have been described in detail in an earlier section (1.2.2). In HBM, secretory cells were commonly found up to 110 days postpartum, while the ductal cells were rarely encountered after eight days (Brooker 1980). It has been suggested that the presence of these epithelial cells in milk provides a means of evacuating dead cells which have reached the end of their secretory life although through trypan blue exclusion, it was found that more than 90% of these cells are viable (Gaffney 1982; Thompson et al. 1998). Whether these epithelial cells are present in HBM purely due to mechanical sloughing during milk expression or due to a yet-unknown functional role, similar to the leukocytes present are an intriguing question worth pursuing. In addition to the biological reasoning behind their existence, epithelial cells from HBM may have several functional uses: the establishment of cell lines for lactogenesis studies whereby they can constitute an in vitro system for the study of proliferation and differentiation of mammary epithelial cells as well as application in

cancer research as most human breast tumours arise from the mammary epithelial cells that line the milk duct and milk-secreting-cells (Buehring 1972).

1.4 Adult Stem Cells

Stem cells are defined by their functional attributes. Typically, one would define the stem cells of a particular tissue as undifferentiated cells capable of proliferation, producing a large number of differentiated functional progeny and yet able to self-maintain the population by exhibiting self-renewal. Stem cells can be subcategorized into cloned cells, embryonic, fetal and adult, depending on the source. Each of these has its own set of advantages and disadvantages of which ethical and legal concerns have been raised by cloning (Smaglik 2001; 2002; Check 2002), and the use of embryonic or fetal stem cells (Antoniou 2001). Adult stem cells, while avoiding the ethical and legal issues, however are believed to have much restricted potential. Historically, many adult tissues were thought incapable of regeneration until cells with regenerative capability were found in most adult tissues including the liver (Sell 1978; Thorgeirsson 1996; Michalopoulos and DeFrances 1997), intestines (Potten and Loeffler 1990), retina (Tropepe et al. 2000), skin (Watt 1998), muscle (Seale and Rudnicki 2000), central nervous system (Gage 2000), mammary glands (Ormerod and Rudland 1986) and others. Stem cells from adult tissues, now proven present, are still believed to be restricted in their capacity to produce cells from tissues other than the one they arose in (Slayton and Spangrude 2004). However, a number of studies have since challenged this view (Horwitz et al. 1999; Kaufman and Ildstad 1999; Krivit et al. 1999; Morroni et al. 2004), indicating that adult stem cells from various organs are actually highly plastic, and that they can differentiate into not only cells of their original source tissue, but also cells of unrelated tissue and even germ layers. These studies range from demonstrating

bone marrow-derived cells forming hepatocytes (Petersen et al. 1999), myocytes (Gussoni et al. 1999) and even neurons (Mezey et al. 2000). Apart from the highly plastic bone marrow-derived cells, reversible transdifferentiation between the epithelial cells and adipocytes in the mammary gland have also been established (Morroni et al. 2004).

In conclusion, adult stem cell biology is a front runner in the emerging field of regenerative medicine and in the following sections, two particular adult stem cells that are of relevance to my research question will be reviewed in detail.

1.4.1 Mammary Stem Cells

Mammary gland development and function would not be possible without tissue-specific stem cells. The cycle of pregnancy-associated proliferation, differentiation, apoptosis, and remodelling, which can occur several times during the entire lifespan, can only be explained by the presence of a long-lived population of stem cells with a near-unlimited capacity to generate functional cells (Anderson and Clarke 2004).

1.4.1.1 Evidence for Their Existence

Although the existence of MaSC have long been postulated, it was not until 1959 when Deome demonstrated the reconstitution of mammary gland when isolated mammary epithelium from donor mice were transplanted into cleared mammary fat pads (Deome et al. 1959). The existence of MaSC was then further verified when Ormerod and team demonstrates that fragments of mouse mammary epithelium can give rise to complete glands when transplanted into the fat pads of syngeneic hosts cleared of their endogenous epithelium (Ormerod and Rudland 1986). Limiting dilution studies in

which clones derived from single cells could be identified on the basis of their patterns of mouse mammary tumour virus viral integration have shown that an entire fully functional mammary gland could be derived from a single cell and that this single cell can give rise to both luminal epithelial and myoepithelial cell types, suggesting the presence of a self-renewing pluripotent stem cell in the mouse mammary gland (Kordon and Smith 1998). This is further affirmed by Shackleton and team who demonstrated the reconstitution of a functional mammary gland capable of producing milk proteins by a single stem cell (Shackleton et al. 2006), proving conclusively their presence.

1.4.1.2 Location of the Putative Stem Cells

It has been suggested that the cap cells of the TEBs in the mouse mammary glands during the pubertal development contain the population of multipotent stem cells. This postulation is based on the observation that these cells have a high mitotic rate and that they are capable of following two different fates: either they enter the body of the TEB and become luminal epithelial cells, or they migrate across along the outmost stratum of the attached duct to become myoepithelial cells (Anderson and Clarke 2004). Although the human mammary gland has a TEB-like structure during pubertal development, it does not have a similar arrangement of cap and body cells, making it unclear whether the population of stem cells reside in the TEB-like structure (Anderson and Clarke 2004).

It was postulated that the MaSC should be those intermediate between the epithelial and myoepithelial cells which occur in growing ductal structures, particularly the TEBs. This is because they are able to give rise to both ductal epithelial cells and myoepithelial, the latter by an irreversible process (Rudland et al. 1997). The former

ductal epithelial cells subsequently differentiate normally into alveolar cells in a reversible manner or under certain pathological conditions give rise to squamous cells (Rudland et al. 1997). In studies conducted by other groups, it was shown that mixed colonies were only obtained when CK8- and CK18-positive cells were plated and never when common acute lymphoblastic leukaemia antigen (CALLA)-positive myoepithelial cells were used (Stingl et al. 1998). Collectively, this suggests that the most likely candidates for stem cells in the mammary gland reside alongside the luminal epithelial cell population, and not within the myoepithelial cell population (Anderson and Clarke 2004).

1.4.1.3 Characterisation of Mammary Epithelial Stem Cells

Early searches for mammary-specific stem cell markers were based on the observations of Smith and Medina that mammary epithelial explants contained pale or light-staining cells, and that only these cells, which exist both in small and large forms enter mitosis when placed in culture (Smith and Medina 1988; Chepko and Smith 1997). Small light cells were described as mammary epithelial stem cells based on the presence of mitotic chromosomes, lack of any specialized organelles, and their basal location within the gland. Their nuclei were also found to contain dense clumps of heterochromatin. These cells were present in side-by-side homogenous pairs as well as heterogeneous pairs, suggesting their ability to undergo symmetric and asymmetric division (Welm et al. 2003). Morphological studies of the developing implants using immunocytochemical and ultrastructural techniques have suggested the presence of these stem cell populations in the basal cell layers of ducts and end-bud. The cells populations may be the basal clear cells (Smith and Medina 1988) or a cap cell type intermediate in morphology between epithelial and myoepithelial cells (Williams and Daniel 1983;

Ormerod and Rudland 1986; Rudland 1991). The location of the stem/ progenitor cells was further affirmed by a more recent study where cells of the regenerative compartment of the mammary gland, which is also the basal epithelial layer stained positive for nestin (Li et al. 2007).

Various methodologies have been employed to identify and characterise these mammary stem/ progenitor cells but *bona fide* mammary stem cell markers have remained elusive. In mice, it was discovered that stem cell antigen (Sca-1)–positive cells are able to reconstitute mammary glands but since this antigen is not detectable in human tissues, other MaSC markers need to be found and used in place of Sca-1 and as yet, there is no conclusive marker for mammary stem cell, with several markers suggested by different research groups. Several studies have described bi-potent progenitors cells in normal adult mammary tissue based on the sorting with epithelial specific antigen (ESA), mucin-1 (MUC-1) and α6-integrin with cells that are MUC-1-/weak/ESA+/CD10+/weak/α6-integrin+ were able to generate clones containing cells of both lineages (Stingl et al. 1998; Stingl et al. 2001). Gudjonsson and team reported that cells that give rise to mixed colonies comprising both luminal and myoepithelial cells expressed CK19 and are $CK14^{+}CK18^{+}$ (Gudjonsson et al. 2002a) while Dontu et al and Bocker et al in 2 separate research articles report that CK5-positive cells define the stem cell population (Bocker et al. 2002; Dontu et al. 2003a). Mammary stem/ progenitor cells were also found enriched in oestrogen receptor (ER)-positive populations (Gudjonsson et al. 2002a; Dontu et al. 2003b; Clarke et al. 2005).

Using flow cytometric cell sorting based on exclusion of the fluorescent DNA dye Hoechst 33342, a method that has crossed over from the haemopoietic system, 3

different groups have a range between 0.2% (Alvi et al. 2003; Clayton et al. 2004) and 1% (Dontu et al. 2003a), and as high as 5% (Clarke et al. 2003; Clarke et al. 2005) of side-population (SP) being present in human breast tissue and that only cells within this SP can produce colonies with both myoepithelial and luminal epithelial cell types (Dontu et al. 2003a; Clayton et al. 2004; Clarke et al. 2005). Drawing results from these murine models, it was then found that SP cells give rise to all three mammary epithelial cell lineages when transplanted into cleared fat pads (Welm et al. 2002; Alvi et al. 2003) which further support the hypothesis that the SP is enriched in mammary epithelial stem cells. Finally, human SP cells seem to form much larger branching colonies in matrigel compared to non-SP (NSP) (Clarke et al. 2005) and they are highly enriched for the ability to grow in non-adherent conditions as spheroids, another feature of stem/progenitor cells (Dontu et al. 2003a), mentioned in detail below.

In addition to surface markers and proteins, another less biased approach is the isolation and propagation of the mammary stem and progenitor cell using a suspension medium; this is a method extrapolated from the field of neural stem cells which are routinely grown as neurospheres (Campos 2004; Suzuki et al. 2004). For this method of isolation through culture, it was found that human mammary epithelial cells form spherical colonies at clonal densities in the serum-free medium, exhibiting a property of stem cells (Dontu et al. 2003a; Dontu et al. 2004; Dontu and Wicha 2005). Dontu et al further establish that only SP and not NSP are able to form the spherical colonies, also known as mammospheres (Dontu et al. 2003a). Extending the use of mammospheres' culture to identify MaSC, the latter were found enriched in $Lin^{-}/CD24^{+}/CD29^{hi}$ or $Lin^{-}/CD24^{+}/CD49f^{++}$ population (Shackleton et al. 2006; Stingl et al. 2006a) and that the cells that formed the mammospheres are capable of self-renewing and they differentiate

into terminal ductal lobular units (TDLU) when placed into matrigel-coated plates (Dontu et al. 2003a). Shackleton and team also went on to quantify the frequency of mouse MaSC which was approximately 0.1% of the cells in freshly prepared cell suspension from mammary tissue (Shackleton et al. 2006). They also showed through flow cytometry that mammary gland of both mice and human contain a parallel distribution of basal and luminal subpopulations and that in both species, the most primitive cell types identified thus far are those that copurify with basal cells that express the highest level of CD49f and lower levels of CD24 (for mice) or EPCAM (for humans) (Stingl et al. 2006b).

The observation that three types of colonies (luminal only, myoepithelial only and both cell types) can be formed from single mammary epithelial cells when they were plated at low densities (Stingl et al. 1998; Gudjonsson et al. 2002b) indicating that there are the luminal restricted and the myoepithelial restricted progenitors and the stem cells that are bipotent (Anderson and Clarke 2004).

It was found that MaSC reach senescence after 40 to 50 self-renewing stem cell divisions (Kordon and Smith 1998). They exhibit ductal growth senescence on serial transplantation after six generations (Young et al. 1971; Smith et al. 2002).

Stem cells are found in the mammary gland during all stages of development and very interestingly, there even exists a population of stem cells which are parity-induced (Wagner et al. 2002). Using transgenic whey acidic protein-driven Cre and Rosa 26-fl-LacZ mice, Smith and colleagues demonstrated the presence of a new mammary epithelial cell population which originates from the differentiating cells during

pregnancy and that 5-10% of this parity-induced epithelium survives post-lactational involution after the first pregnancy. With successive pregnancies, the population percentage increases, reaching 60% of the total epithelium in multiparous females (Wagner et al. 2002; Henry et al. 2004; Boulanger et al. 2005). These cells served as progenitors and differentiate into both ductal and lobuloalveolar cells upon serial transplantation. They, similar to the known MaSC, proliferated to produce both luminal and myoepithelial cells in limiting dilution transplantation (Boulanger et al. 2005). This unique population of cells is implicated as a crucial factor in the observed risks of parous females in contracting breast cancers.

1.4.1.4 Association with Tumorigenesis

As stem cells persist in the mammary gland throughout reproductive life, they have the potential to accumulate genetic damage and to transmit it to their progeny and hence are regarded as prime targets for oncogenic transformation (Anderson and Clarke 2004). This is supported by increasing evidence of the presence of cancer stem cell in malignancies of various tissues (Reya et al. 2001; Smith 2002; Dontu et al. 2003b; Smalley and Ashworth 2003; Al-Hajj and Clarke 2004; Waterworth 2004). This together with the observation that majority of the human breast tumours arose in the TDLU and that they are luminal cells (Wellings et al. 1975; Taylor-Papadimitriou et al. 1986; Taylor-Papadimitriou et al. 1989) brings about a theory that breast cancer may be at least in part, a stem cell disease.

Nestin is expressed on the regenerative component of the mammary gland. Interestingly, breast tumours with a basal epithelial phenotype, which is highly aggressive and poorly differentiated, also express nestin in all of the sixteen tumours tested by Li et al. (Li et

al. 2007). Global transcriptional profiling have found that the tumours within the basal breast cancer subtype are particularly associated with mutations in BRCA1 (van 't Veer et al. 2002) and Li et al further showed that nestin expression is well correlated with BRCA1 associated cancer, which are classified as a basal breast cancer (Li et al. 2007). This suggests that at least one cancer subtype, the basal breast cancer display a progenitor-like phenotype (Li et al. 2007), once again implicating MaSC as an aetiological factor in breast cancer.

Malignant mammary epithelial cells are unable to differentiate to the two mature cell types in culture (Rudland et al. 1997), which can possibly be explained by two different theories. In the first theory, there are two different types of epithelial stem cells in normal mammary glands, one which can differentiate to benign lesions and the other which give rise to malignant lesions. This was illustrated with the immunocytochemical staining pattern of the epithelial cells within the normal human mammary gland where there was a differential expression of CK19, with the malignant cells showing an increased expression (Taylor-Papadimitriou et al. 1983; Taylor-Papadimitriou and Lane 1987). An alternative theory states that the ability of the epithelial stem cells to differentiate is impaired when they become malignant (Rudland et al. 1997), which is supported by evidence obtained from experimental carcinomas in rats and observations made on human carcinomas. In the rodent studies, the benign tumours which contain predominantly only the one major epithelial cell type can develop into malignant metasising tumours with serial transplantation (Rudland 1987) whereas in the human, transfection of rat epithelial stem cells lines with cells of malignant origin will induce specifically the malignant cell types and not differentiate into myoepithelial-like or alveolar-like cells (Davies et al. 1993).

The above evidences strongly suggest the involvement of MaSC in tumourigenesis and therefore, the study of mechanism of self-renewal, the malignant transformation as well as the potential cell lineages that can arise from MaSC are likely to provide new insights into the carcinogenic process. The realisation that proper cellular differentiation is a powerful inhibitor of cancer initiation provides a strong rationale for pursuing the identification of the stem cells susceptible to carcinogenesis and of the genes that control this process. This would allow us to target key pathways to either induce differentiation or apoptosis, improving the current state of the art treatment regimen (Kalirai and Clarke 2006). In addition, this approach may allow the development of new tools for developing rational strategies for breast cancer prevention (Russo et al. 2005; Cariati and Purushotham 2008).

1.4.2 Haemopoietic Stem and Progenitor Cells

Haemopoietic stem cells is the first stem cell source used for transplantation purposes and has been in use over 30 years (Clark et al. 2003). HSC transplantation, after myeloablative chemotherapy in both autologous and allogeneic settings have become an established therapeutic option in the management of haematological malignancies, thalassaemia, sickle cell disease and aplastic anaemia (Vassiliou et al. 2001; Tabbara et al. 2002). In fact in recent years, research on HSC suggests that their therapeutic uses extend beyond haematological diseases. There have been many studies that illustrate the multi-lineage stem cell plasticity of HSC as they differentiate and contribute to the liver, (Lagasse et al. 2000; Forbes et al. 2002), central nervous system (Mezey et al. 2000), endothelia (Grant et al. 2002) and skeletal muscle (Bittner et al. 1999). All these collectively suggest the theoretical potential for HSC to treat many diseases. While ex vivo expansion of HSC may allow their sources to be viable sources of stem cells for

transplantation purposes and replacement therapies, it is necessary to understand that much is still unknown about HSC and hence, the interest in HSC, particularly its plasticity has to continue and this interest is very likely to be sustained.

Haemopoietic stem cells (HSC) are multipotent precursors that have self-renewal capacity and the ability to regenerate all the different cell types that comprise the blood forming system which include erythrocytes, neutrophils, monocytes, macrophages, platelets, T lymphocytes, B lymphocytes, natural killer T cells, dendritic cells and others (Bonnet 2002; McCulloch and Till 2005; Broxmeyer et al. 2006). Limiting dilutions analysis of total bone marrow preparations allowed quantitative estimation of HSC frequencies ranging from 1 in 10,000 to 1 in 100,000 (Harrison 1980; Szilvassy et al. 1990; Harrison et al. 1993). The primitive HSC is by definition, both self-renewing and capable of long-term repopulation (LTR) and resides at the base of progenitor cell development (Lord 1997). It must have the ability to give rise to all lineages of mature blood cells and offer radioprotection to a lethally irradiated host (Nolta and Kohn 1997; Lagasse et al. 2000).

Haemopoietic progenitor cells, on the other hand, have more limited capacity for self-renewal than stem cells, and they are more restricted in their capacity to give rise to multiple cell types, with the hierarchy as shown in Fig. 1-2. However, more is known about them than HSC. This is in part because the stem cells are rarer in frequency than the progenitors, and assays for progenitor cells performed in vitro, are easier to do than assays for engrafting stem cells, which are done in vivo (Broxmeyer et al. 2006). Progenitors are ranked as those that are immature with enhanced proliferative capacity and those with that are more mature, with decreased proliferative capacity, and those

that are mature, with decreased proliferative and more limited and restricted differentiation capability (Broxmeyer et al. 2006). It is believed that the immature subsets of progenitors are responsive to stimulation by combinations of growth factors (Shaheen and Broxmeyer 2005). Thus multipotential progenitor cells (termed colony-forming unit-granulocyte, erythrocytes, macrophage, megakaryocytes, CFU-GEMM or CFU-Mix) are denoted by the colonies of mixed lineage blood cells they give rise to in semisolid culture medium when the cells are stimulated in vitro, with combinations of cytokines such as erythropoietin (Epo), stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3), in the absence or presence of thrombopoietin (TPO) (Broxmeyer et al. 2006).

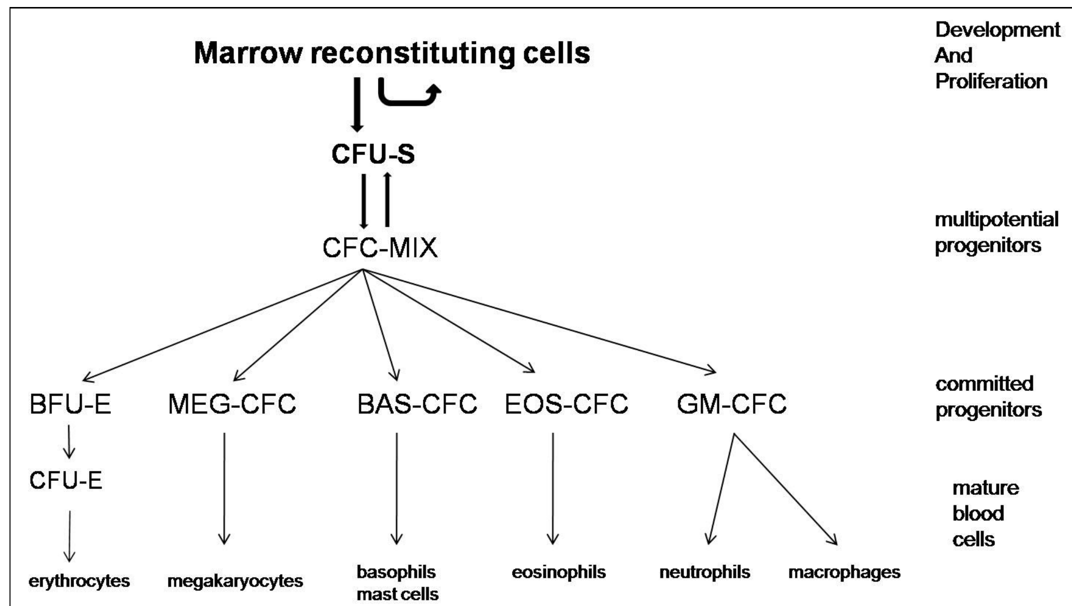


Figure 1-2 **Scheme of Haemopoiesis.** *Adapted from (Lord 1997).*

1.4.2.1 Sources of Haemopoietic Stem/ Progenitor Cells

Both cord blood and bone marrow are well-established sources of haemopoietic stem/ progenitor cells. Haematopoietic progenitor cells in cord blood are increased in frequency compared with those in bone marrow (Broxmeyer et al. 1989) and are enhanced in proliferative capacity, generation of progeny and also replating capacity in vitro (Broxmeyer et al. 1989; Carow et al. 1991; Broxmeyer et al. 1992; Cardoso et al. 1993; Lansdorp et al. 1993; Lu et al. 1993). Transplantation of HSC forms the basis of consolidation therapy in cancer treatments and they are used to cure or ameliorate a number of haematologic and genetic disorders (Shizuru et al. 2005; Steward and Jarisch 2005). As such, these cells are widely harvested from cord blood (Broxmeyer 2004; Broxmeyer 2005) and bone marrow for therapeutic purposes.

1.4.2.2 Isolation and Purification of Haemopoietic Stem/ Progenitor Cells

The rarity of the primitive HSC results in the difficulty in their isolation and purification. The most commonly used technique for separating early progenitor cells today is based on the use of monoclonal antibodies. Once labelled with antibody, cells can be separated using solid phase immunological methods of immunomagnetic particles or flow cytometry (Lord 1997). One such antigen which identifies HSC is CD34 (Link et al. 1996), which make up approximately 1% of all human bone marrow cells (Hao et al. 1995). Subsequently, by assessing the coexpression with other cell surface markers, it is possible to obtain cell fractions that are more highly enriched for primitive cell types (Craig et al. 1993; Huang and Terstappen 1994; Hao et al. 1995). It was found that lin-/CD34+ subpopulation have defined more primitive precursors with haemopoietic repopulating activity that expresses combinations of the CD59 surface antigen related to Stem-cell antigen (Sca-1), the vascular endothelial growth factor receptor-2 (VEGFR2) and low levels of c-kit (CD117), Thy-1 (CD90) and CD38 (Baum et al. 1992; Gunji et al. 1993; Civin et al. 1996; Hill et al. 1996; Kawashima et al. 1996; Larochelle et al. 1996; Ziegler et al. 1999). It was only in 1997 when CD133 become appreciated as another important HSC marker (Yin et al. 1997; de Wynter et al. 1998; Gallacher et al. 2000; Lang et al. 2004; Hess et al. 2006). CD133, the human homologue of mouse Prominin-1 (Shmelkov et al. 2005) was first identified as a selective human HSC surface molecule. Selection for CD133+ haemopoietic precursors yields more than 90% CD34+ cells while containing all the human repopulating activity (Hawley et al. 2006). The lin-/ CD34+ or CD34+/ Thy-1+/ Lin- or CD34+/ CD38- or CD133+ purified cell types were capable of engraftment into SCID mice (Chen et al. 1994; Murray et al. 1995; Kerre et al. 2001; Handgretinger et al. 2003).

Besides the use of cell surface markers, rhodamine 123 which preferentially accumulated in active mitochondria) and Hoechst 33342 (a bis-benzimidazole that binds to the adenine -thymine-rich regions of the minor groove of DNA) are two fluorescent dyes that have been routinely used to characterize haemopoietic precursor populations (Visser et al. 1981; Bertoncello et al. 1985; McAlister et al. 1990; Wolf et al. 1993; Leemhuis et al. 1996). Goodell et al described a method that simultaneously monitors the low fluorescence intensity of Hoechst 33342 staining at 450nm and at >650nm after ultraviolet excitation, which identifies a rare subpopulation referred to as side-population (SP) cells enriched with HSC (Goodell et al. 1996). First used, on mouse bone marrow cells, it was found to contain the majority of long-term haemopoietic repopulating activity (Goodell et al. 1996). Since then, the SP assay has been applied to human haemopoietic tissues (Goodell et al. 1997; Storms et al. 2000; Uchida et al. 2001; Preffer et al. 2002; Eaker et al. 2004; Naylor et al. 2005) and this list is not comprehensive.

1.5 Discussion

MaSC have been isolated in both human and murine models (Dontu et al. 2003a; Shackleton et al. 2006). They have been suggested as the likely targets for malignant transformation leading to breast cancer which remains the most common malignancy among females worldwide. This is due to common properties of longevity and the self-renewal property with tumorigenic cells (Dontu et al. 2003b; Dontu et al. 2005; Ponti et al. 2005). Research groups worldwide are attempting to identify the cell type most susceptible to malignancy (Dontu et al. 2005; Sleeman et al. 2006). The identification of susceptible cell types may allow the control of this life-threatening disease through

primary prevention. Identification of mammary stem cell markers will also help us in understanding normal development of the mammary gland, and provide tools to characterize the role of stem cells in breast cancer. To date, the only source of human MaSC were tissues derived from reduction mammoplasties (Dontu et al. 2003a; Shackleton et al. 2006; Stingl et al. 2006b). Isolation of mammary stem/ progenitor cells from mammary tissue requires a complicated isolation technique with long digestion times by various enzymes. This is due to the fact that the composition of the mammary gland contains adipocytes, collagen-impregnated stroma surrounding ductal epithelium, basement membrane, and tightly interacting epithelial cells, which does not make it an ideal tissue to produce single cell suspension efficiently. In order to do so, long digestive procedures are required which may potentially destroy and/ or mask cell surface epitopes important for isolation and identification of these cells (Wagner et al. 2002; Welm et al. 2003). Therefore, there is a demand for other sources of MaSC which are more easily available and obtained in a less invasive manner and do not require cumbersome procedures to derive single cell suspension for subsequent work.

The work revolving around HBM thus far has mainly been focusing on the nutritive value for the infant. Immune and epithelial cells are the only cell types reported to exist in HBM [discussed in detail in Section 1.3.6]. Until now, there has been no literature reporting the presence of mammary stem/ progenitor cells in HBM, although there was a commentary highlighting the possibility and that it could be an unappreciated source of stem cells (McGregor and Rogo 2006). Relating to other systems with epithelial cell lining, there have been some studies documenting the presence of the epithelial stem cell in the luminal discharges due to the proximity of the stem cell niche to their luminal cavities. Most recently, mesenchymal progenitor cell types have been isolated through

the collection of human menstrual blood (Hida et al. 2008; Musina et al. 2008), which has been postulated to derive from the stromal regions of the endometrial glands. Similarly, it has been proposed that the epithelial stem cells reside in the niche at the base of the glands in the endometrium (Gargett 2007), and shown to be present just beneath the luminal epithelium and in the endometrial-myometrial junction (Chan and Gargett 2006; Cervello et al. 2007). From urine, rare stem/ progenitor cell types from the epithelial, urothelial and smooth muscle lineage have been identified at a clonal level, with the capacity for self-renewal and multi-lineage differentiation (Zhang et al. 2008). In the gastro-intestinal system, stem cells have been localized to the basal crypts (Marshman et al. 2002; Bjerknes and Cheng 2006), although there have been no reports of these epithelial stem cells being shed into the gastrointestinal tract. Nonetheless, extrapolating these reports to a tissue of common lineage, they suggest the plausibility of stem/ progenitor cells being shed into HBM either by means of sloughing or active shedding into the lumen for yet unknown purposes.

I aimed to identify and isolate putative stem/ progenitor cells which have shed into HBM during lactation, although whether this shedding serves a specific functional purpose sets the platform for another research question. Pursuing the question of whether stem cells exist in HBM would also allow the complete identification of the components in HBM which might elucidate other potential powerful biologic benefits of human lactation (McGregor and Rogo 2006). From Section 1.3.6, it has been established that majority of the cellular component of HBM are leukocytes (early days postpartum). As HSC share the common markers of CD133 and SP with MaSC, the possibility that these cells may be true HSC needs to be explored, although the

biological rationale for such a finding is still an unknown entity. Hence, I have devoted some energy in exploring this possibility in HBM.

1.5.1 Hypotheses

1. Adult stem/ progenitor cells are present in HBM.

2. These stem cells are able to demonstrate multi-lineage differentiation capacities.

1.5.2 Significance of Study

This attempt to isolate stem cells from human milk may have various implications relating to:

1. Breast tissue engineering - This will be of value to patients who have had mastectomies or traumatic injuries, where autologous stored stem cells could be used as a tissue engineering cellular source.

2. Elucidating mechanisms for mutagenesis and oncogenesis in breast cancer - It has recently been shown that cancers, including those in the breast (Al-Hajj et al. 2003), arise from transformed stem cells which accounted for the heterogeneity of solid tumours, and their resistance to chemotherapy. Thus, the availability of MaSC will allow studies of mutagenesis and other events related to carcinogenesis to be studied in detail, and possible therapeutic agents to be developed.

3. Biology of lactation - Until now, it is unclear how the process of lactation is initiated and maintained at the cellular and glandular level. Identification of MaSC may allow the dissection of cellular response to changing hormonal signals which occurs during pregnancy and lactation. This may have implications in identifying causes for various lactational problems, an issue with dire consequences particularly in developing countries (Mepham 1983).

4. Dissecting microchimerism of maternal cells in breastfed infant - If mammary and/ or haemopoietic stem cells are actively passed out to the infant, we could be potentially studying the effects of these cells in the breastfed infant which might explain in part, the long term benefits HBM have over formula, and certainly open up a new field of study worth pursuing.

5. Providing a non-invasive novel source of HSC - This potentially allows the banking of HSC from all lactating females for their future use, given the known fact that HSC transplantation has been applied to several clinical settings where they have been proven to be beneficial to the recipients (Vassiliou et al. 2001; Tabbara et al. 2002).

2 Materials and Methods

2.1 Samples

2.1.1 Research Ethics Board Approval

Ethics approval was obtained from the Domain Specific Review Board (DSRB) of the National Healthcare Group (NHG) for collection of human samples (DSRB Ref: D/04/206). All participants gave written informed consent for the donation of samples for research purposes.

2.1.2 Cells from Human Breast Milk

Informed written consent were obtained from healthy lactating women who had delivered within a year and the breast milk samples collected in sterile containers. A total of forty-one breastfeeding women (duration of lactation – 1 to 12months) were recruited to participate in the study. All samples were collected and processed within four hours of expression.

Whole cell population was isolated from human breast milk by centrifugation. The expressed human breast milk was spun down at 400g for 10mins. The cell pellet was then washed twice with RPMI medium before use for subsequent experiments.

2.1.3 Umbilical Cord and Peripheral Blood Cells

Umbilical cord blood was collected at normal deliveries of full term pregnancies (n=2) while peripheral blood were collected from a volunteer (n=1). The umbilical cord blood as well as the peripheral blood was diluted 4-fold with PBS (137mM NaCl, 10mM Phosphate, 2.7mM KCl, pH 7.4) with 2mM EDTA (Gibco). Every 35mL of diluted

blood was carefully layered over 15mL of Ficoll 1077 (Invitrogen, California, USA) and centrifuged at 400g for 35mins in a swinging-bucket rotor with brakes off. The top layer of plasma was removed before collecting the mononuclear cells at the interphase with a 22 Gauge needle attached to a 3mL syringe. The cells were washed twice with PBS/ 2 mM EDTA before being used as controls; cells from cord blood were used for methylcellulose assays and cells from peripheral blood for reverse transcription polymerase chain reaction; these are discussed more in detail in Sections 2.2.6 and 2.3.

2.1.4 Cells from Fetal Brain

Human fetal brain tissue samples from medically aborted fetuses at 15-18weeks of gestation were collected (n=5). The hippocampus, subventricular zone, thalamus, brain stem, cerebellum, spinal cord, anterior and posterior cerebra were teased out and mechanically minced with a scalpel. The minced tissues were then dissociated enzymatically with 0.25% trypsin for 15mins at 37°C. Thereafter, an equal volume of 40mg/ mL of bovine serum albumin (BSA) suspended in Earles' balanced salt solution (Invitrogen, Carlsbad, CA) were added to inactivate the trypsin. The cell suspension is then filtered through 70µm filters (BD Biosciences, Franklin Lakes, NJ) and washed twice with PBS (137mM NaCl, 10mM Phosphate, 2.7mM KCl, pH 7.4) before counting. Viability of cells was determined with 3% acetic acid with methylene blue (StemCell Tech, Canada). Cells isolated from fetal brain were used for reverse transcription polymerase chain reaction and as one of the positive controls for spherical cultures.

2.1.5 Fetal Mesenchymal Stem Cells

Mesenchymal stem cells were derived by flushing the bone marrow of medically aborted fetuses at 18weeks (n=1). This is done using a 22 Gauge needle attached to a syringe. The suspension is then filtered through 70μm nylon filter (BD Falcon, MA, USA) to get single cells. These fetal mesenchymal stem cells were used for reverse transcription polymerase chain reaction in Section 3.3.2.

2.1.6 Breast Adenocarcinoma Cell Line, MCF-7

MCF-7, a breast adenocarcinoma cell line was obtained from American Tissues Culture (ATCC, HTB - 22). This cell line is derived from a 69 year old Caucasian lady. This cell line is chosen as a suitable control for my work as it retains several characteristics of differentiated mammary epithelium. In addition, it has been reported to contain a group of cells known as side-population which possesses the ability to exclude Hoechst dye actively and these cells exhibit clonogenicity, a property of stem cells (Cariati et al. 2008).

2.2 Cell Culture

2.2.1 Epithelial Cell Culture

The cellular fraction of breast milk was suspended and cultured in RPMI (Roswell Park Medical Institute) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 2mM L-glutamine, 4μg/ mL insulin (Sigma-Aldrich, Mossouri, USA), 0.5μg/ mL hydrocortisone (Sigma-Aldrich), 20ng/ mL epidermal growth factor (BD Bioscience, California, USA), 60ng/mL cholera toxin (Sigma-Aldrich) and 0.1mg/ mL

of penicillin/streptomycin/fungizone (Biowhittaker, Verviers, Belgium). The cells were cultured for 7 to 50 days at a density of 10^5 cells/ cm^2 until confluence. Cells cultured for immunocytochemistry were cultured on 13mm diameter circular coverslips in 24-well tissue culture plates (Sarstedt, Numbrecht, Germany) at 37°C with 5% CO_2 and 95% relative humidity.

Epithelial medium placed onto an established culture of lactocytes for 72hours were collected and passed through 0.2µm filters to remove cells. They were then kept in aliquots at -20°C till used as conditioned medium for optimisation of mammosphere medium subsequently.

2.2.2 Mammosphere Culture

To attempt to grow MaSC from cells directly isolated from HBM, cells from HBM were also grown in a serum-free mammary epithelial growth medium (Cambrex, New Jersey, United States), supplemented with B27 (Invitrogen), 20ng/ mL epidermal growth factor (Sigma-Aldrich), 20ng/ mL basic fibroblast growth factor (BD Biosciences) and 4µg/ mL heparin (Sigma-Aldrich). Cells were placed on ultra-low attachment plates at various densities of 1, 100, 1,000 and 3,000 cells per well of a 96-well plate to establish their ability to grow clonally, in a manner MaSC from human mammary tissue were previously cultured (Dontu et al. 2003a). These cultures are then observed weekly after 2 weeks, up to 5 weeks for mammospheres formation.

2.2.2.1 Optimisation of Mammosphere Media

Cells intended for growth into mammopsheres were first plated singly into each well of a 96-well plate. I next proceeded to plate the cells into low adherence 96-well plates at both low plating density of $3x10^3$cells/ cm^2 and high plating density of $9x10^3$cells/ cm^2.

Further to this, I added in conditioned medium, derived as described in Section 2.2.1 in varying concentrations as shown in Table 2-1. Varying concentrations of epidermal growth factor (EGF) (Sigma-Alrich), basic fibroblast growth factor (bFGF) (Sigma-Aldrich) and fetal bovine serum (FBS), as tabulated in Table 2-1, were also used in the optimisation of mammosphere medium for culture.

Substances added into mammosphere medium					
Fetal Bovine Serum (%)	1	3	5	10	20
Epidermal Growth Factor (ng/ ml)	20	40	60	80	100
Basic Fibroblastic Growth Factor (ng/ ml)	20	40	60	80	100
Conditioned Medium (%)	1	5	20	50	80

Table 2-1: **Factors for optimisation of mammosphere medium.**

2.2.2.2 Coating of Substratum

2.2.2.2.1 Matrigel^{TM} coating

Matrigel^{TM} Matrix (BD Biosciences) is a solubilized basement membrane preparation extracted from sarcoma, a tumor rich in extracellular matrix. At room temperature, it produces biologically active matrix material resembling the mammalian cellular base and is known to be especially suited for culture of epithelial cells.

Matrigel™ is allowed to defrost slowly on ice. 20µl of matrigel was aliquoted and spread with a pipette tip into each well of a 96-well plate (Nunc, Roskilde, Denmark). The excess was aspirated away and the plates left to incubate on ice for 30mins followed by incubation at 37°C for 30mins to ensure setting of the gel. Thereafter, 200µl of medium was added into each well.

2.2.2.2.2 Collagen coating

Twenty microliters of 1mg/ mL collagen solution (Sigma-Aldrich) was placed onto each well of a 96-well plate and spread using a pipette tip. The plate was left at room temperature to air-dry after aspirating away the excess collagen. 200µL of medium was then added into each well and the plate left at 37°C for an hour, before use.

2.2.2.2.3 Gelatin coating

Dissolve 12mg of gelatin (Sigma-Aldrich) in 12mL of sterile water. The 0.1% gelatin solution was sterile-filtered before being loaded onto each well of a 96-well plate. The plate was then left in the incubator at 37°C for an hour, before the excess gelatin solution was removed. 200µL of medium was then added into each well.

2.2.2.2.4 Fibronectin coating

The recommended use of fibronectin (Sigma-Aldrich) is at a concentration of 1.5µg/ cm^2 and each well of a 96-well plate has an area of $0.32cm^2$. Therefore, 8µL of 0.1% fibronectin was loaded onto each well. After coating the wells, the plate was left in the incubator at 37°C for an hour before 200µL of mammopshere medium was added into each well.

2.2.3 Neurosphere Culture

For neurospheres initiating assays, cells isolated from the fetal brain were plated at a concentration of $3x10^4$ cells per mL of neurosphere medium which is 1:1 Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/ F-12) (Gibco) supplemented with 1% N2 supplement (Gibco) and 1x antibiotic/antimycotic (Invitrogen) for four weeks.

At the end of four weeks, the neurospheres were then dissociated using TrypIE (Invitrogen) and mechanical dissociation. The single cells are then cultured with differentiation medium, DMEM/F-12 with 1% N2 supplement, 1% FBS and 1x antibiotic/antimycotic on poly-L-ornithine–coated coverslips for one week before the coverslips were washed with phosphate buffered saline (PBS) and fixed with 1:1 methanol/ acetone for 5mins at -20°C. These were then used for immunocytochemistry.

2.2.3.1 Poly-L-ornithine Coating of Coverslips

Each coverslip was placed into a well of a 24-well plate and washed with 70% ethanol and left to dry. Thereafter, each glass coverslip was covered with 200µL of 0.1mg/ ml poly-L-ornithine solution for two hours at room temperature. The solution was then aspirated away and wells washed with distilled water twice. The 24-well plates were left to dry completely overnight in the incubator before storage at 4°C for use within a week.

2.2.4 Fetal Mesenchymal Stem Cell Culture

These cells which were flushed out from bone marrow were cultured in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco), supplemented with 10% FBS (Sigma-Aldrich), 1% L-glutamine (Gibco) and 1x penicillin/ streptomycin (Gibco) in T_{75} flasks, at a cell density of 10^6 cells per mL of culture media. The non-adherent cells were removed after three days and new medium added. The adherent cells were subcultured every two to three days when confluent, at a cell density of 10^4 cells per cm^2 in T_{75} flasks. Adequate cell numbers were cultured and then harvested for RNA extraction, to be used as a positive control in Section 3.3.2.

2.2.5 Culture of Cell Line, MCF-7

These cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco), supplemented with 10% FBS (Sigma-Aldrich), 1% L-glutamine (Gibco) and 1x Penicillin/ Streptomycin (Gibco) in T_{75} flasks. The cells were subcultured 1:3 every four to five days when confluent. MCF-7 cells were used as both negative [in Section 3.3.1, 3.3.2 and 3.3.3] and positive controls [in Section 3.3.4 and 5.4.1.1.2].

2.2.6 Methylcellulose Culture

Cells derived from cord blood and HBM were placed in HSC-CFU lite with Epo (Miltenyi) at 1,000 to 3,000 cells per mL as per manufacturer’s instructions. Specifically, individual specimens were mixed with 3 mL of methylcellulose containing bovine serum, stem cell factor, granulocyte-macrophage colony-stimulating factor, interleukins-3 and 6, erythropoietin, and plated in 35-mm tissue culture dishes in duplicates for two weeks and then observed for colonies formation.

2.3 Reverse Transcription Polymerase Chain Reaction

2.3.1 RNA Extraction

Whole RNA was extracted from cells using RNAeasy columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The isolated DNAse-treated RNA was quantified with a spectrophotometer (Beckman, California, USA).

2.3.2 Reverse Transcription

One microgram of total RNA was reverse-transcribed using oligo-dT primers (Proligo, Sigma-Aldrich), RNase inhibitor (Roche, Basel, Switzerland) and the Sensiscript kit (Qiagen) at 25°C for 15mins, 42°C for 60mins and 72°C for 15mins. Negative controls, without reverse transcriptase, were performed for each RNA sample to ensure absence of DNA contamination.

2.3.3 Polymerase Chain Reaction

PCR cycling conditions were as follows: 94°C for 120s followed by 30-40 cycles of denaturation (94°C for 15-30s), annealing (55-68°C for 15-30s) and extension (72°C for 60s) and a final extension at 72°C for 4mins. The PCR products were electrophoresed in 1.2% agarose gels stained with ethidium bromide in TE electrophoresis buffer. The PCR bands were visualised using a UV illuminator. RT without reverse transcriptase and PCR reaction with water in place of cDNA were negative controls to exclude DNA contamination. Commercial genomic DNA (Applied Biosystems, California, USA) was used as a positive control. Primers used for PCR amplification were as shown in Table 2-2.

Primer	Sense 5'→3'	Antisense 5'→3'	Size (bp)
GAPDH	aaggactcatgaccacagtccatg	ttgatggtacatgacaaggtgcgg	673
NES	ggtcagttcctgaagttcactcag	cctagtactatcgggattcagctg	343
CK5	cgacaaggtgcggttcctg	gcagattggcgcactg	683
CK14	gatgacttccgcaccaagtatgag	tcaatctccaggttctgcatggtg	440
CK18	agaaatctgaaggccagcttggag	taccctgcttctgctggcttaatg	372
CD34	gacactgtggacttggtcaccag	gaggaggaagccatggagatcag	310
CD133	ccaagttctacctcatgtttgg	accaacagggagattgcaaagc	480
ON	atttgatgatggtgcagaggaa	ggtggttctggcagggattt	65
ALP	caggctggagatggacaagttc	ggacctgggcattggtgtt	68
OP	cctgccagcaaccgaagtt	cactatcacctcggccatca	67
MSI	gtgtgaggtgcttaacctatcagc	acagatgtggagagaagagacacc	359
NFM	cttcagccagtcctcgtccc	tcctccaggtggtccgagtc	312

Table 2-2: **Primers for PCR.**

2.4 Immunostaining

2.4.1 Slides and Controls

Cells were either cytospun onto glass slides at a density of 2-5x10^4 cells per slide or grown on glass coverslips in 24-well plates till sub-confluency.

2.4.1.1 Controls for Immunocytochemistry

Controls for immunocytochemistry include an external negative cell type known not to express the antigen, an external positive cell type known to express the antigen and an internal negative control, where the incubation with primary antibody is omitted.

2.4.1.2 Controls for Flow Cytometry

Isotype controls were used for the directly conjugated antibody. Incubation with the secondary antibody alone was used as control for those antigens detected via indirect flow cytometry. An external cell type known to express the antigen-in-study and an

external cell type known to be negative for the antigen-in-study were used to verify protocol.

2.4.2 Immunocytochemistry

A list of antibodies and concentrations used are provided in Table 2-3. Prior to staining, samples were rinsed with PBS and fixed with ice-cold 1:1 acetone-methanol for 10min. After fixation, potential non-specific binding was blocked with 10% goat serum (Vector Laboratories, California, United States) and 3% Bovine Serum Albumin (Sigma-Aldrich) in Tris-buffered saline for an hour at room temperature. For immunocytochemistry, all incubations were done in a humidified chamber.

2.4.2.1 Immunocytochemistry by Alkaline Phosphatase/ Horse Radish Peroxidase

Cells were incubated with primary antibody overnight at 4°C for 2hours at room temperature and washed thrice with PBST (phosphate- buffered saline with 0.5% Tween-20), 5mins per wash. Thereafter, they were incubated with biotinylated secondary antibody (1:100) for 30mins at room temperature, washed thrice with PBST before incubation with streptavidin alkaline phosphatase (Vector Laboratories) or streptavidin horseradish peroxidase (Dakocytomation, Glostrup, Denmark) (1:100) for 30mins at room temperature. Freshly-prepared substrate was then added and incubated for 10mins in the dark for colour reaction to occur. Following this, the slides were rinsed with distilled water, followed by absolute ethanol. Depex mounting medium (Electron Microscopy Sciences, Pennsylvania, USA) was used to seal the coverslips.

2.4.2.2 Immunocytochemistry by Immunofluorescence

Coverslips were incubated with primary antibody overnight at 4°C and followed by incubation with a fluorescent-labelled secondary antibody for 30mins at room temperature in the dark. The slides were then washed thrice with PBS and rinsed with distilled water. HardSet mounting medium with DAPI (Vector Laboratories) was added and the cells visualised and photographed with a Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss, New York, USA).

2.4.3 Flow Cytometry

Flow cytometry is used to measure the numbers of particles or cells as they flow in a fluid stream singly through a detection point. The important feature of flow cytometric analysis is that measurements are made on individual particles or cells within the suspension and not as average values for the whole cell population. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) conjugated antibodies were used in the experiments. The emission maximum of fluorescein is 530nm and is visualised as green. PE has an emission maximum of 578nm and is visualised as red. Indirect flow cytometry was also performed with the use of anti-mouse IgG AlexaFluor 488 as the secondary antibody. The emission maximum of AlexaFluor 488 is similar to that of FITC.

Cells were enzymatically lifted by incubation in trypsin for 5mins at 37°C, washed, and re-suspended in 100µL of blocking solution comprising 2mM EDTA in PBS supplemented with 10% FBS and 10% goat serum. After 30mins, antibody solution was added. Cells were washed twice in PBS by centrifugation at 400g for 10mins. Secondary labelling was carried out when unconjugated primary antibodies were used.

This process involved incubation of the cells with a 10% solution of secondary antibody in PBS for 20mins in the dark. Washing was then carried out to remove unbound labels. Cells were fixed by incubation in 4% buffered formaldehyde for 15mins, followed by washing in PBS. All stained samples were analysed with Cyan^{TM} ADP analyzer (Dakocytomation) within 72hrs and the results analysed with software, Summit 4.2.

2.4.3.1 Extracellular Antigens

For directly labelled antibodies, cells were suspended in 90μL of staining buffer and 10μL of fluorochrome-labelled antibody added into the suspension. After an incubation of 20mins in the dark, the cells were washed twice with staining buffer (0.5% BSA/ 2mM EDTA in PBS) by centrifugation at 400g for 5mins. The cells were re-suspended in 1 mL staining buffer for analysis.

For the indirectly-labelled antibodies, cells have to undergo a blocking step by incubation with 5% bovine serum albumin (Sigma-Aldrich), 20% goat's serum (Vector Laboratories) for 45mins at room temperature. Cells were then incubated with primary antibody diluted 20 fold with blocking solution. The cells were incubated for 30mins at room temperature before washing twice with staining buffer (similar to above). Cells were re-suspended and incubated for 15mins at room temperature in the dark in 488 AlexaFluor-labelled goat anti-mouse antibody: blocking solution (1:200). The cells were then washed twice with staining buffer before re-suspension in 1 mL of staining buffer for analysis.

2.4.3.2 Intracellular Antigens

Each cell sample was suspended in 1ml PBS, with 2ml 4% paraformaldehdye (Integrated Contracted Manufacturing Pte Ltd, Singapore, Singapore). The cell suspensions were washed with staining buffer and cells spun down by centrifugation. Cell pellets were incubated with 1mL of methanol for 10mins at -20°C before washing twice with staining buffer. The cells were then incubated in 1mL of 0.5% Nonidet P40 (Roche) in blocking solution (as mentioned above) for 45mins at room temperature to permeabilise the cells. Primary antibody was added and incubated for 30mins at room temperature in the dark. Cell pellet were then washed twice with staining buffer and re-suspended for incubation at room temperature in the dark with 488 AlexaFluor-labelled goat anti-mouse antibody diluted 200 fold with blocking solution. The cells were washed twice with the staining buffer before re-suspension in 1mL of staining buffer for analysis.

Antibody reactive to	Host species	Dilutions used		Manufacturer
		ICC	FACS	
CK5	Mouse	1:50	1:20	Sigma-Aldrich
CK14	Rabbit	1:100	1:20	Chemicon
CK18	Mouse	1:100	1:20	Chemicon
CK19	Mouse	1:100	1:20	Dako
Nestin	Mouse	1:100	1:20	Chemicon
Nestin	Rabbit	1:200	NA	Chemicon
594 anti-Rabbit	Goat	1:400	1:10	Molecular Probes
488 Anti-Mouse	Goat	1:400	1:10	Molecular Probes
CD133	Mouse	NA	1:10	R&D Systems
CD34	Mouse	NA	1:10	Chemicon
RPE-CD24	Mouse	NA	1:10	BD Pharmingen
FITC-CD29	Mouse	NA	1:10	Chemicon
CD49f	Mouse	NA	1:10	Chemicon
CDw338	Mouse	NA	1:10	StemCell
Stro-1	Mouse	NA	1:25	Chemicon
RPE-CD117	Mouse	NA	1:10	Dako
GFAP	Rabbit	1:200	NA	Sigma-Aldrich
BIII Tubulin	Mouse	1:100	NA	Chemicon
PDGFRα	Rabbit	1:100	NA	Upstate
RPE- IgG2a	Mouse	NA	1:10	Dako
FITC- IgG2b	Mouse	NA	1:10	Dako
RPE- IgG1	Mouse	NA	1:10	Dako
FITC- IgG1	Mouse	NA	1:10	Dako
PE- IgG2b	Mouse	NA	1:10	Miltenyi

Table 2-3: **List of antibodies and concentrations.**

2.5 Cell Sorting

2.5.1 CD133 tagged Dynabeads™

Mouse Antihuman-CD45 (BD Pharmingen, California, United States) and antihuman-CD133 monoclonal antibody (Miltenyi, Gladbach, Germany) were tagged onto Dynabeads™ (Dynal AS, Invitrogen) and used to isolate CD45+ or CD133+ cells from

the whole cell population in HBM on separate occasions. Dynabeads^{TM} are first washed with PBS/ 0.1% BSA. Using a magnet, the Dynabeads^{TM} are then separated and supernatant discarded. Mouse antihuman CD133 or mouse antihuman CD45 were incubated with the Dynabeads^{TM} are a concentration of 0.5µg per 10^7 Dynabeads^{TM} for an hour at 4°C with gentle tilting and rotation. Thereafter, the tube is placed into a magnet for 2mins, to separate the Dynabeads^{TM} from the supernatant which is discarded. The Dynabeads^{TM}, now attached with relevant antibody are washed thrice with PBS/ 0.1% BSA before use. Isolation of the CD133+ or CD45+ cells from HBM were carried out based on manufacturer's instructions. Purified mouse IgG (Chemicon) were bound to Dynabeads^{TM} and used as negative controls.

2.5.2 Fluorescence Activated Cell Sorting

2.5.2.1 Hoechst Dye Exclusion

Hoechst staining was performed as previously described (Storms et al. 2000). Cells from HBM, cord blood, and cell line, MCF-7 were sorted. Cells were then suspended in culture medium with Hoechst 33342 (Sigma-Aldrich) at a final concentration of 2.5µg/ mL and incubated for an hour at 37°C. In the control samples, verapamil (Sigma-Aldrich) was added to a final concentration of 50µg/ mL. After incubation, samples were washed and re-suspended in Hanks Balanced Salt Solution (HBSS)(Gibco) supplemented with 5% FBS (Gibco). Cell populations were stained with 1µg/ mL propidium iodide (Sigma-Aldrich) for exclusion of dead cells in analysis. Flow cytometry was performed using FACStarPLUS (Becton Dickinson, New Jersey, USA) and FACSAria (Becton Dickinson) and the analysis done using Summit 4.2 and FACSDiva respectively.

2.5.2.2 CD133

Cells from HBM and cord blood were incubated with phycoerythin conjugated CD133/2 antibody (Miltenyi) diluted 1:10 in staining buffer (0.5% BSA/ 2mM EDTA in PBS). The cells are washed twice with staining buffer after incubation for 20mins at 37°C and then suspended at 10^6cells/ mL of staining buffer for sorting. Isotype controls were in place and all samples were analyzed and sorted by flow cytometry using FACSAria and the software, FACS diva (Becton Dickinson).

2.6 Proliferation Studies Using AlamarBlue®

AlamarBlue® is a commercially available reagent used as an indicator of cellular health of the cells-of-interest. Viable cells in culture are able to convert resazurin, the active ingredient in alamarBlue® to resorufin. This is reflected as colour change from the nonfluorescence blue resazurin to the bright red fluorescent resorufin.

AlamarBlue® is used as per manufacturer's instructions, and incubated with the cells for four hours before absorbance is read at 590nm filter with Spectrophotometer (Beckmann, California, United States)

2.7 Statistical Analysis

Data were first checked for compliance to normal distribution. Parametric data were shown as mean ± standard error mean (SEM) and analysed using Student's t-tests and one-way ANOVA where applicable. Non-parametric data were shown as median ± SEM and analysed using Kruskal-Wallis where applicable. A p-value of <0.05 was considered significant.

3 Cellular Component of Expressed Human Breast Milk

3.1 Introduction

Research in HBM has largely been focused on its nutritional value to the breastfed infant. As such, one can find numerous research articles on the nutrients like carbohydrates, proteins, lipids and vitamins (Mata and Wyatt 1971; Bracco et al. 1972; Ebner and Schanbacher 1974; Picciano and Guthrie 1976; Siimes et al. 1979; Packard 1982; Bitman et al. 1983; Davies 1983; Howell et al. 1986; Morriss 1986; Goldman and Goldblum 1989; Jensen 1989; Canfield et al. 1991; Hamosh 1995; Koldovsky 1995; Lammi-Keefe 1995; Coppa et al. 1999). On the other hand, research in the cellular component of HBM is a field less explored (Crago et al. 1979; Ho et al. 1979; Brooker 1980; Davies 1983; Wirt et al. 1992; Xanthou 1997; Thompson et al. 1998).

Working with colostrum donated from fifty-four healthy donors, Crago and team found a range of 1.1×10^5 to 1.2×10^7 cells per mL of colostrum (Crago et al. 1979). This cellular component comprises 30-47% macrophages, 40-60% polymorphonuclear leucocytes, 5.2-8.9% lymphocytes, and 1.3-2.8% colostral corpuscles. The team found a great variability in the total number of cells and distribution of various cell types both among individuals and also in the same individual studied longitudinally, although no significant increase or decrease in total number of cells per mL of colostrum were found (Crago et al. 1979).

In the following year, Brooker studied the composition of HBM up to 110 days postpartum. He examined HBM derived from 30 lactating women and found that the population of intact cells was relatively low, greatly outnumbered by the membrane-bound cytoplasmic fragments. In addition the immune cells, lymphocytes, macrophages and polymorphonuclear leukocytes have garnered more interest due to their role in

conferring immune protection to the neonate against pathogens (Brooker 1980). The different cell types have been found to transfer cell-mediated immunity (Mohr 1973; Parmely et al. 1977), produce IgA antibodies (Murillo and Goldman 1970) and interferon (Emodi and Just 1974), and engulf enteric pathogens (Pitt et al. 1977). The epithelial cells on the other hand are subjected to much lesser scrutiny. He found the presence of three distinct epithelial cell types: secretory cells denoted by the presence of abundant cisternae of rough endoplasmic reticulum, lipid droplets and a Golgi apparatus, squamous epithelial cells which are derived from the stratified squamous epithelium of the galactophores and/ or the skin of the nipple and lastly, ductal cells which are found in clusters of two to four cells and they occur in small numbers (Brooker 1980). He also established that the secretory cells are commonly found up to 110 days postpartum whereas the duct cells are found only up to eight days postpartum.

In this chapter, I aim to extend this body of work by examining the cellular component of HBM beyond one year postpartum in detail, which is more than 3 times the duration which Brooker et al has explored. HBM is dynamic in nature and examination of the cellular component of HBM beyond 110 days will allow us to explore the cellular changes beyond the first 3 months of lactation. I will also investigate the cell types by applying more modern stem cell techniques, based upon the surface markers that they possess.

3.2 Cellular Content

In order to reduce the possiblity of cellular death post expression of HBM, all the samples were processed within four hours post-collection. The cell concentration in milk ranges widely from 10^3 cells/ mL milk to $8x10^5$ cells/ mL milk, with a mean RNA

level of 2.6 ± 0.8 (SEM) pg per cell. No correlation was found between the duration of breastfeeding and cell concentration in HBM, in either inter-individual (n=40, $r^2 = 0.4$) (Figure 3-1) or intra-individual samples (Figure 3-2, n=6, $r^2 < 0.03$). Cellular content of expressed HBM comprised immune cells like neutrophils, basophils, monocytes, lymphocytes and macrophages as well as epithelial cells as published previously (Figure 3-3 & 3-4). A cell type which has not been characterised before was observed. These putatively novel cells were significantly smaller than the immune and epithelial cells, with a high nucleus-cytoplasm ratio as seen on H&E staining (Figure 3-3).

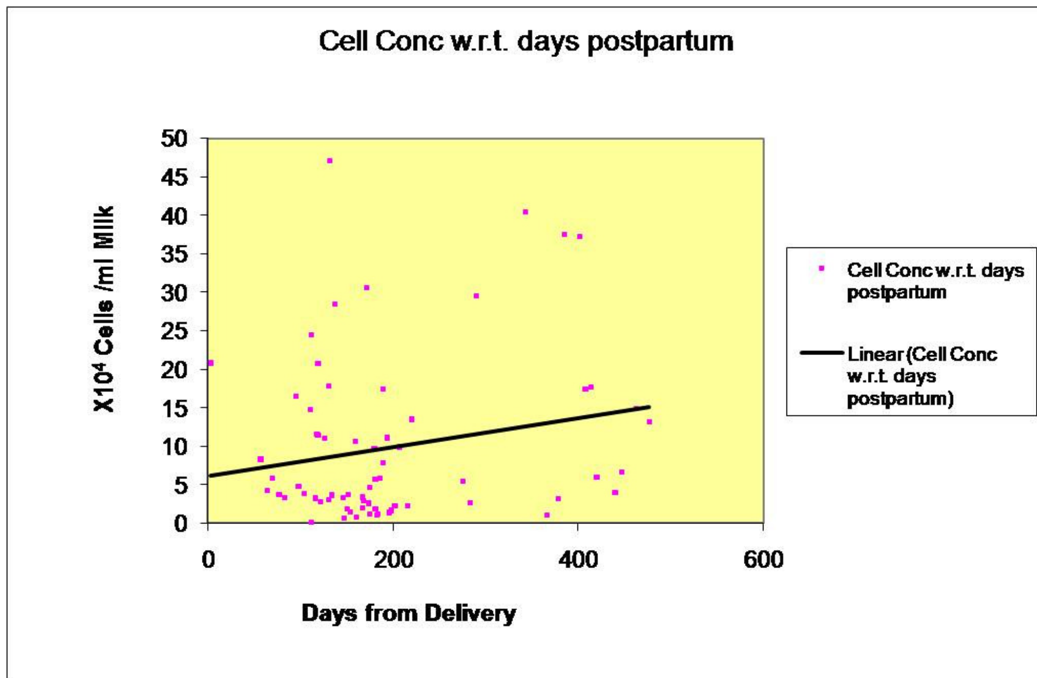


Figure 3-1 Correlation of cell concentration in milk to the duration of breastfeeding. Cell concentrations were not related to the duration of breastfeeding in my study ($R^2 = 0.4$)

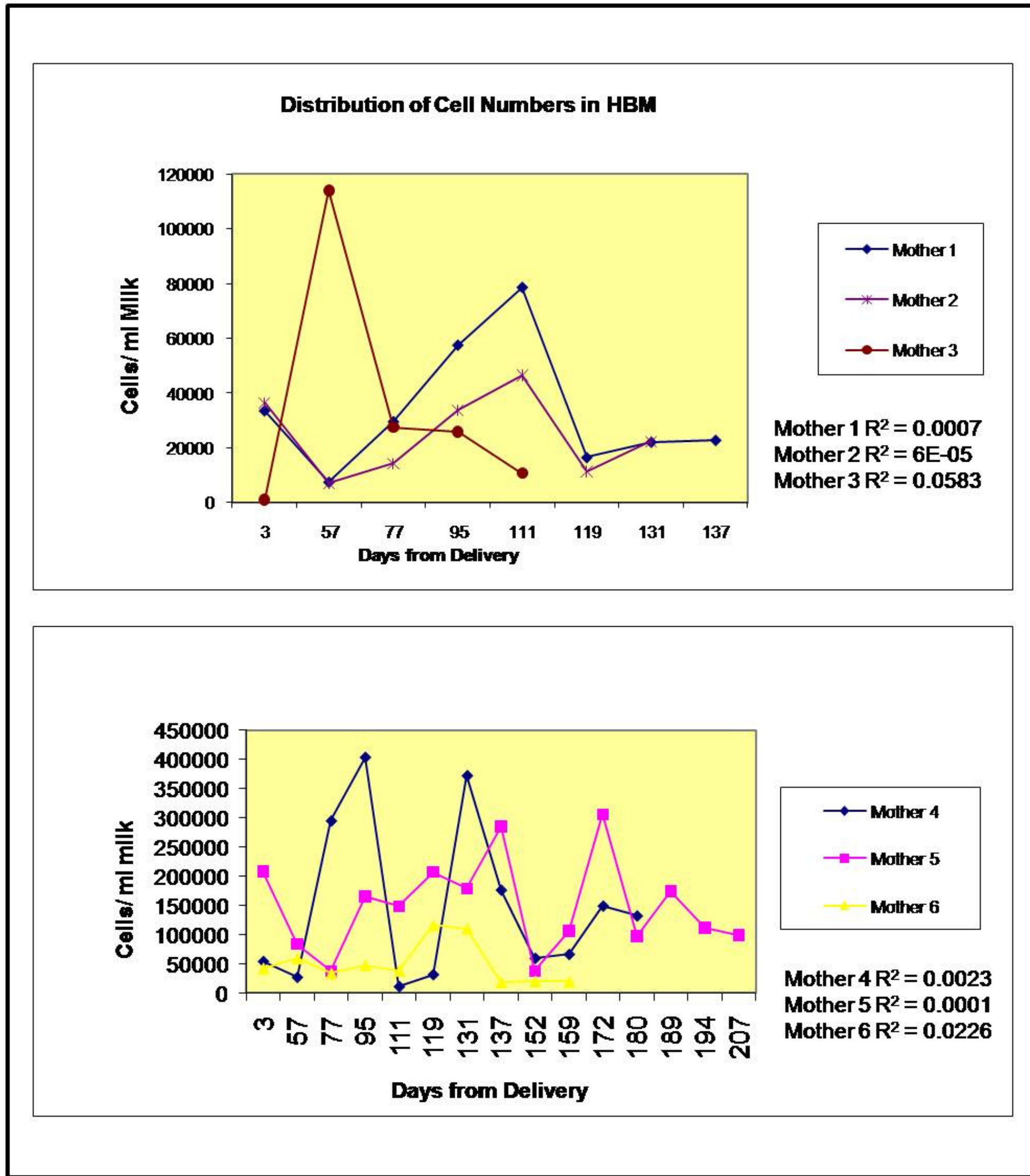


Figure 3-2 Correlation of cell concentration in milk to the duration of breastfeeding in 6 lactating females. Cell concentrations were not related to the duration of breastfeeding in all 6 females when their milk samples are analysed longitudinally ($R^2 < 0.03$).

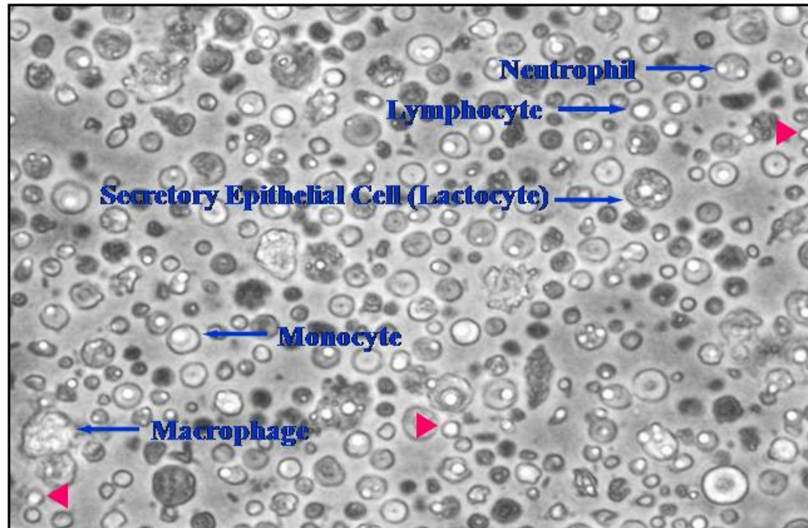


Figure 3-3 Phase contrast image of total cell population in HBM. Neutrophils were recognised by their multi-lobed nucleus. Lymphocytes and monocytes were both circular and were differentiated by the nuclear cytoplasmic ratio. Lymphocytes have a large nucleus with a thin periphery of cytoplasm. The irregularly shaped lactocytes and macrophages were distinguished by the lipid inclusions exclusively found in the lactocytes. A group of cells with a high nuclear cytoplasmic ratio was observed. This group of cells, which are very small, have not been described (red arrowheads).

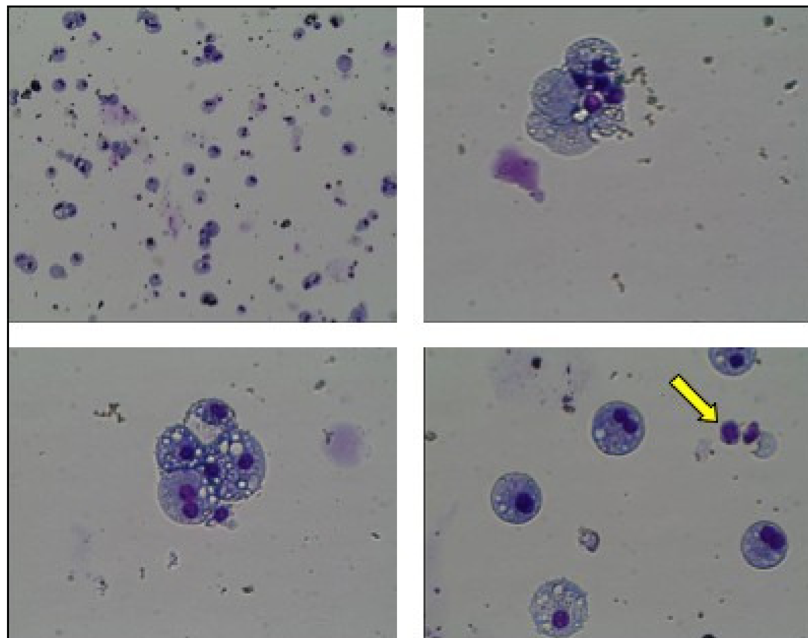


Figure 3-4 Haematoxylin and eosin staining of cells directly spun down from HBM. The epithelial cells shown in high magnification here are larger than lymphocytes (yellow arrow). Numerous intercellular lipid droplets were observed in the epithelial cells.

In order to further characterise the heterogeneous cellular population of HBM, I interrogated the cells for lineage specific markers in this mixed cell population. The presence of haemopoietic, mesenchymal and neural-epithelial lineage specific markers were investigated at both mRNA and protein level.

3.3 Test for Various Lineage Markers

3.3.1 Cell Markers of Haemopoietic Lineage

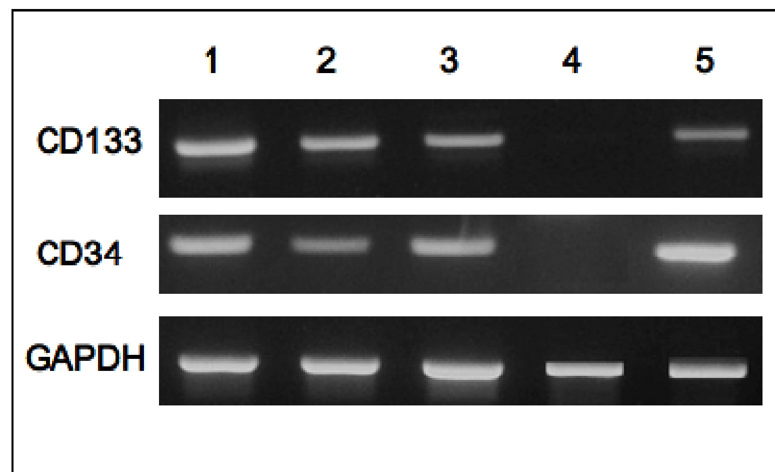


Figure 3-5 RT-PCR of haemopoietic stem markers. mRNA of haemopoietic stem cells (HSC) markers: CD133 and CD34 were present in whole cell population (WCP) of HBM (Lane 1-3). These HSC markers are absent in MCF-7, a breast cancer cell line (Lane 4). Lane 5 displayed mRNA of the HSC's markers on cells isolated from umbilical cord blood, a positive control.

Firstly, I looked for the expression of CD34 and CD133 at the mRNA level. CD34 is a known haemopoietic stem/ progenitor and endothelial cell marker (Fina et al. 1990). CD133 has been associated with a variety of primitive cells in both haemopoietic and neural lineage (Yin et al. 1997). As shown in Figure 3-5, CD34 and CD133 are present in all three milk samples while absent in a breast adenocarcinoma cell line, MCF-7.

This suggests the presence of haemopoietic stem/ progenitor cells in the cellular component of HBM.

Next, I performed immunocytochemistry for CD34 on cytospun cells directly isolated from HBM and found high level staining even in my negative controls (Figure 3-6). This occurred despite employing several techniques to reduce this high level of background staining such as varying the blocking agent from 5% goat serum with 2% BSA to 10% goat serum with 5% BSA, to as high as 50% goat serum and varying the incubation time with the blocking agent from 30mins at room temperature to an hour at 37°C to two hours at room temperature. This high background staining was not observed in other cell types tested, namely the human umbilical vein endothelial cells and erythrocytes (Fig. 3-6c, d). This problem is most likely due to the large amount of cellular debris and lipids present in expressed HBM. Hence, I attempted to optimise the sorting of cells from HBM for immunocytochemistry, as shown in Table 3-1. Despite the several rounds of optimisation, hematoxylin and eosin stained cells derived from cell pellets demonstrated persistence of high background staining (Figure 3-7).

Method	**Procedure Undertaken**
A	Centrifugation at 1,900rpm for 10mins thrice followed by incubation with trypsin/EDTA for 15mins followed by incubation in RPMI for 1.5hrs before cytospun onto slides.
B	Centrifugation at 1,900rpm for 10mins thrice followed by three washes in RPMI before cytospun onto slides.
C	Centrifugation at 1,900rpm for 10mins thrice followed by incubation with trypsin/EDTA for 30mins followed by incubation in RPMI for 2hrs before cytospun onto slides.
D	Centrifugation at 1,900rpm for 10mins thrice followed by incubation with culture medium for 2hrs before cytospun onto slides.

Table 3-1: **Optimisation strategy for isolation of cells for immunocytochemistry.**

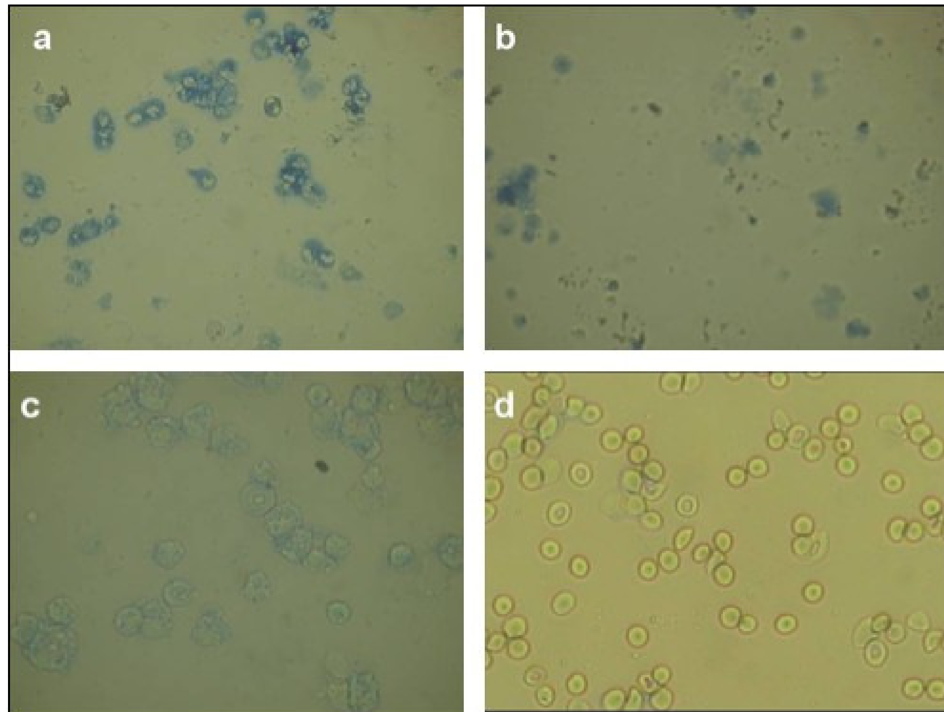


Figure 3-6 CD34 staining. Whole cell population directly isolated from HBM (a-b, b without primary antibody), human umbilical vein endothelial cells (c, positive control) and erythrocytes (d, external negative control). Magnification of 20X.

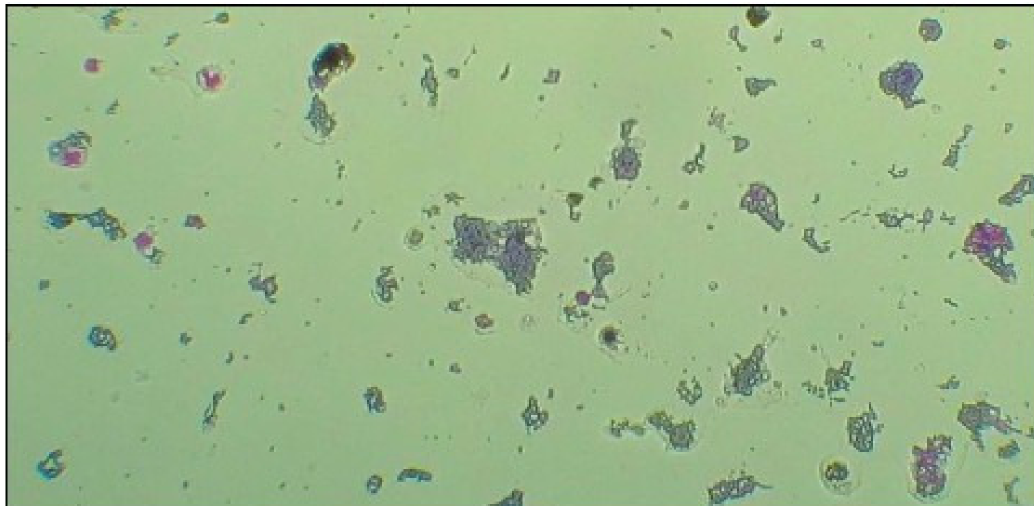


Figure 3-7 Hematoxylin and eosin staining. Whole cell population of HBM stained, with a high amount of debris despite multiple washes. Magnification of 4X.

Following this, I decided to use flow cytometry to investigate the protein level instead. Flow cytometry allows the analysis of a very large number of events, reducing errors of looking at too small a population as in the case of immunocytochemistry. In addition, flow cytometry does not require forceful spinning of cells down (with the debris) while allowing analysis of the cells in suspension. This reduces the clumps present, allowing a more reliable collection of results. The flow cytometer also allows the gating for the exclusion of cellular debris, typically in the bottom left hand corner of a forward-scatter, side-scatter plot. Appropriate isotypes were used to allow robust removal of autofluorescence.

Next, I utilised flow cytometry in an attempt to delineate the cell types found within HBM. Flow cytometry of CD133, CD34 and CD117 were first carried out on a positive control using mononuclear cells (MNC) from umbilical cord blood collected from term pregnancies. In MNC of cord blood, I found 1.12% of the cells to be CD34+, a lower percentage of 0.41% of the cells being CD133+ and lastly, 1.28% of the cells to be c-kit-positive. This is comparable to the published data on the cells present in cord blood (Nimgaonkar et al. 1995). The typical protein profile of the three haemopoietic stem/ progenitor cell markers was as shown (Figure 3-8). By subjecting the WCP of HBM to fluorescence activated cell sorting (FACS), I found a mean of 2.6±0.8% CD133-positive cells, with 1.1±0.15% of the WCP being CD34 positive and 0% of the WCP being c-kit positive (Table 3-2, page 79).

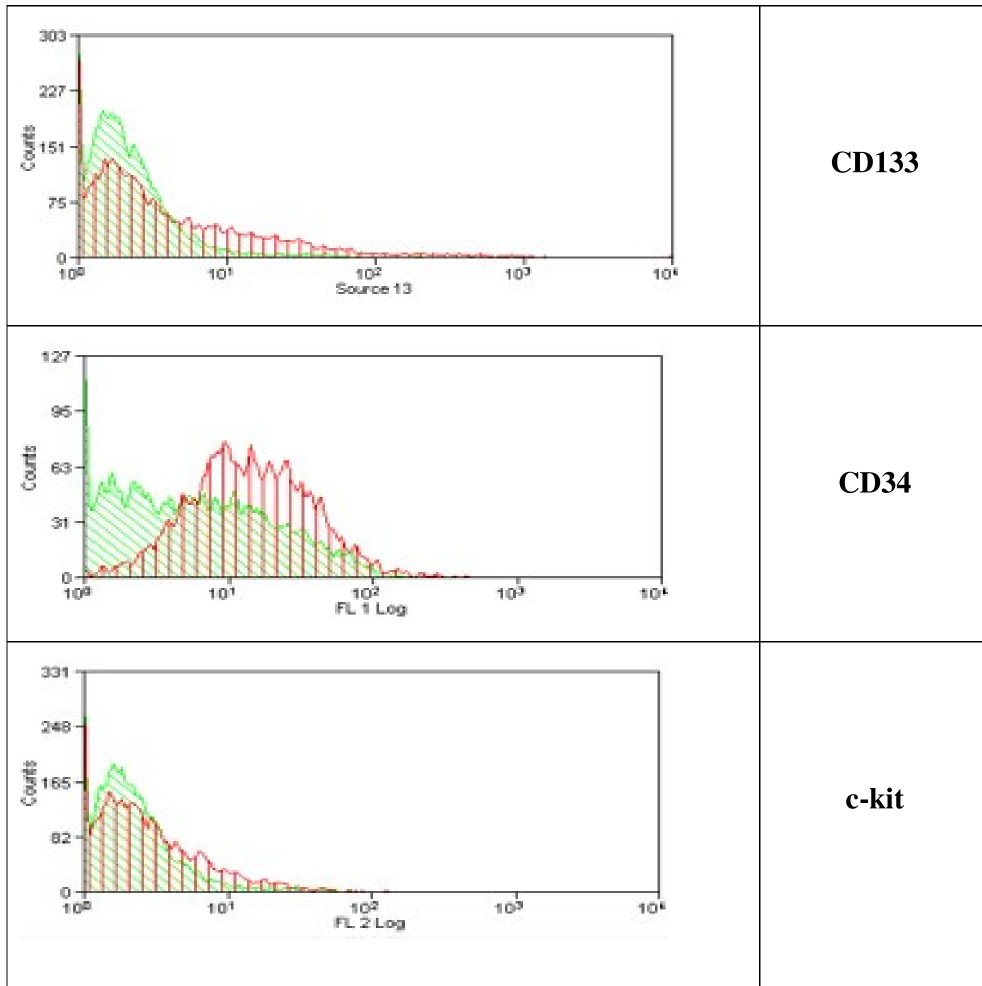


Figure 3-8 Staining for haemopoietic stem/ progenitor markers. WCP directly isolated from HBM are subjected to flow cytometry, analysing 20,000 events for each antigen (red), with an appropriate isotype control (green).

Antigen	Other name	Expression determined by Flow Cytometry		
		Mean %	No. of Samples	SEM
CD133	Prominin-like 1	2.6	4	0.8
CD117	c-kit	0.0	3	0.0
CD 24	BA-1	70.7	3	10.3
CD29	β-1 Integrin	11.5	3	5.8
CD49f	α-6 Integrin	11.4	3	7.3
CDw338	ABCG2	21.1	3	1.8
CD34		0.7	3	0.2
Stro-1		27.8	3	9.1
Nestin		4.7	3	0.2
CK 5		0.9	3	0.2
CK 14		0.2	3	0.2
CK 18		2.0	3	0.1

Table 3-2: **Expression of selected proteins in WCP of HBM.**

3.3.2 Cell Markers of Mesenchymal Lineage

Mesenchymal stem cells are able to differentiate into adipocytes, chrondrocytes and osteocytes (Pittenger et al. 1999). The established mesenchymal stem cells markers are Stro-1, CD105 as well as vimentin. However, CD105 and vimentin are also present on cells of the lympho-haemopoietic system. Specifically, CD105 is present on activated monocytes and macrophages and vimentin on lymphocytes and macrophages (Giorno 1985; Rohde et al. 2006). Therefore, showing their presence on the WCP of HBM would not be indicative of mesenchymal stem/ progenitors in HBM. The immunogen for Stro-1 is still as yet unknown. I therefore decide to run RT-PCR for a terminal cell type of mesenchymal lineage. Three bone markers: osteonectin (ON), alkaline phosphatase (ALP) and osteopontin (OP) were investigated (Figure 3-9). ON, ALP, and OP are markers for early, intermediate and terminal bone respectively (Qi et al. 2003).

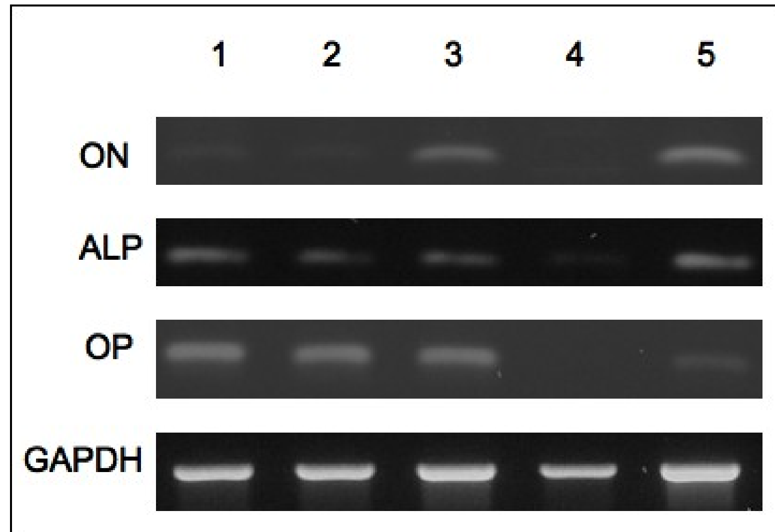


Figure 3-9 RT-PCR for bone markers. ON, ALP and OP were present in WCP of HBM (Lane 1-3). These bone markers were absent in MCF-7 (Lane 4), an external negative control. mRNA from fetal mesenchymal stem cells, an early cell type of the mesenchymal lineage was used as an external positive control (Lane 5).

Fetal mesenchymal stem cell (fMSC) is an early cell type of the mesenchymal lineage and is used as a positive control. An increasing expression of the bone markers, with the lowest expression of the early marker and the highest expression of the late bone marker were noticed in all three samples, shown in Lane 1 to 3.

I performed flow cytometry for Stro -1, an established mesenchymal stem cell marker. I found a mean of 27.8±9.1% of cells which are Stro-1-positive (Table 3-2, page 79).

The RNA present in WCP of HBM containing early bone markers and presence of Stro-1 as determined by flow cytometry, suggest the presence of mesenchymal stem/ progenitor cells in the heterogeneous population. The presence of late bone marker suggests the possibility of terminal mesenchymal cell type like bone being present in the WCP in HBM.

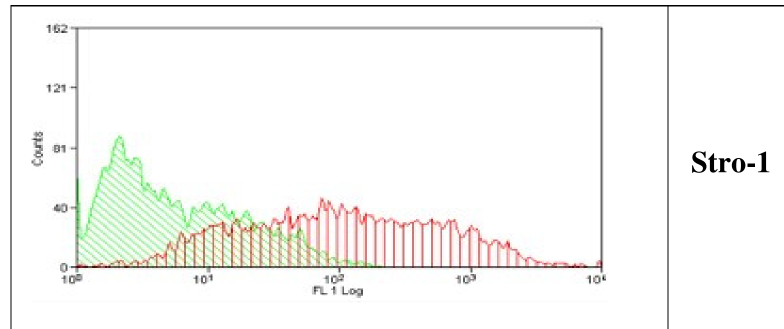


Figure 3-10 Staining for mesenchymal stem/ progenitor markers. Whole cell population directly isolated from HBM and subjected to flow cytometry, analysing 20,000 events for Stro-1 (red), with an appropriate isotype control (green).

3.3.3 Cell Markers of Neural Lineage

From Figure 3-11, it was observed that only nestin is expressed at comparatively high levels. Musashi-1 is a central nervous system (CNS) progenitor cell marker, which is important for determination of mammalian cell fate, maintenance of stem cell state, differentiation and tumorigenesis (Okano et al. 2005). Musashi-1 was found to be expressed in all three samples, although at very low levels in two of the samples (Lane 1 and 2) (Figure 3-11). A similar pattern of gene expression was observed for a differentiated neural cell marker, neurofilament M (NFM), which is typically expressed in neurons (Figure 3-11).

Nestin was expressed in a mean of 4.7±0.2% of cells in HBM by FACS analysis (Table 3-2, page 79).

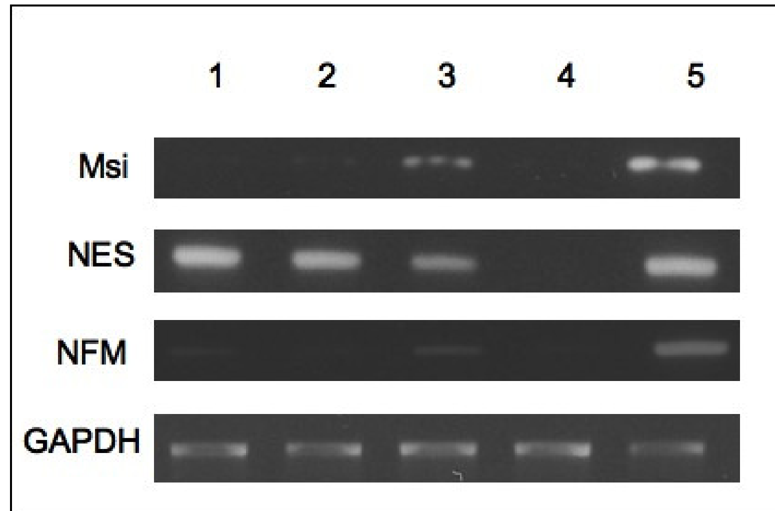


Figure 3-11 RT-PCR for neural markers. Messenger RNA of musashi-1 (Msi), nestin (NES) and neurofilament-M (NFM) were observed in WCP of HBM (Lane 1-3). These neural markers were absent in MCF-7 (Lane 4), an external negative control. RNA directly isolated from human fetal brain was used as a positive control for the neural markers (Lane 5).

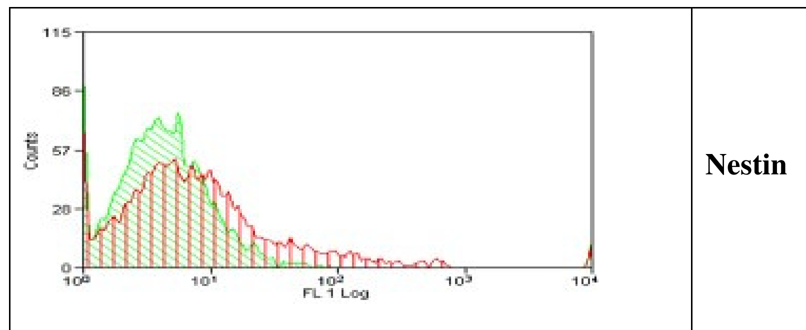


Figure 3-12 Staining for neural stem/ progenitor markers. Whole cell population directly isolated from HBM and subjected to flow cytometry, analyzing 20,000 events for nestin (red), with an appropriate isotype control (green).

3.3.4 Cell Markers of Epithelial Lineage

CK5, CK14 and CK18 are established markers used in characterisation of maturity of mammary epithelial cells. CK5 is a marker for mammary progenitor cells, CK14 is a marker for both mammary progenitor cells and mature myoepithelial cells, and CK18 is an established marker for mature luminal epithelial cells (Bocker et al. 2002). Messenger RNA of all 3 cytokeratins were present at high levels (Figure 3-13).

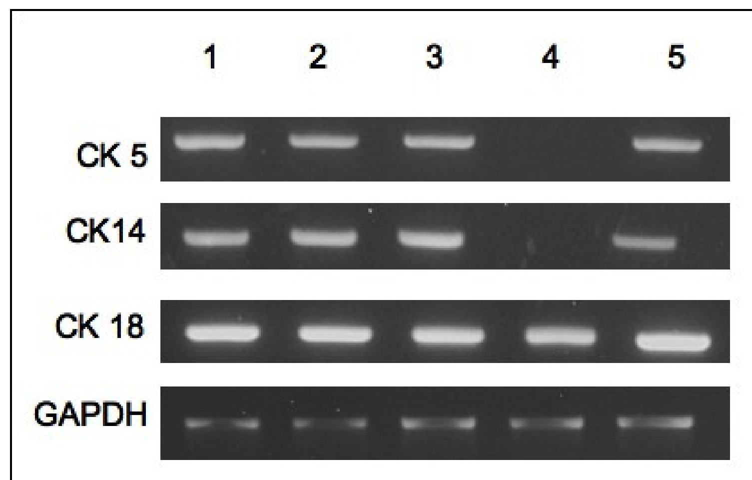


Figure 3-13 RT-PCR for epithelial cell markers. Messenger RNA of CK5, 14 and 18 were present in WCP of HBM (Lane 1-3). The mammary progenitor cell marker, CK5 and myoepithelial cell marker, CK14 were absent in negative control of mononuclear cells in peripheral blood (Lane 4) and present in MCF-7, a positive control (Lane 5).

Flow cytometry showed a mean of 0.93±0.17% CK5+, 0.25±0.16% CK14+ and 1.97±0.05% CK18+ cells in the cellular content of HBM with a typical flow cytometry profile shown in Figure 3-14 (Table 3-2, page 79).

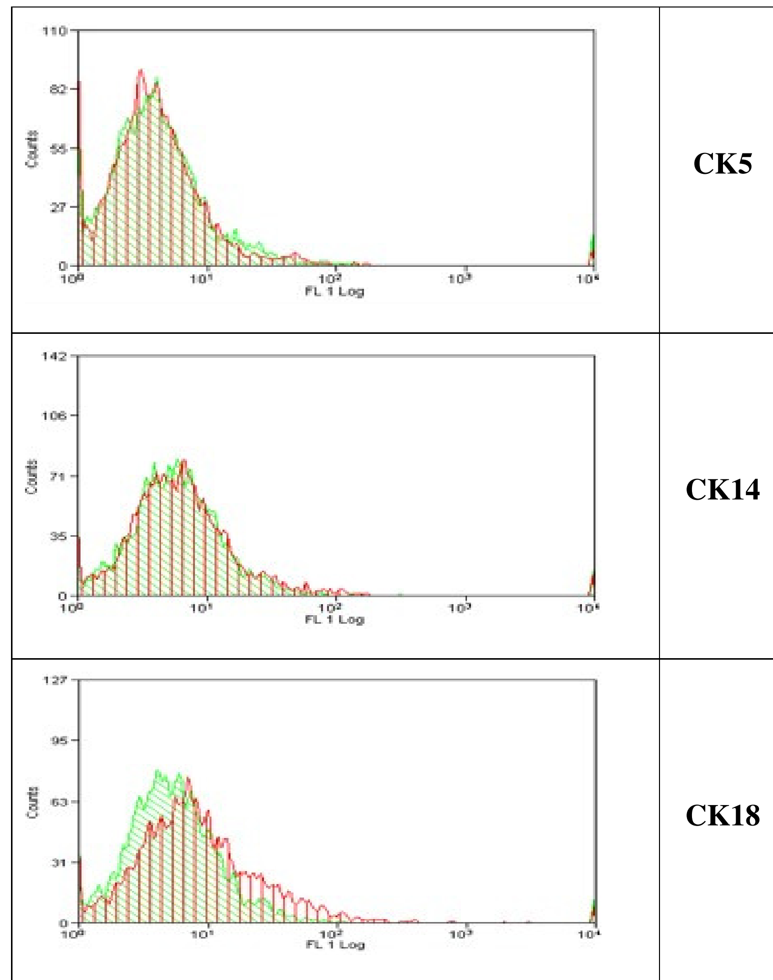


Figure 3-14 Staining for epithelial cell markers. Whole cell population directly isolated from HBM and subjected to flow cytometry, analyzing 20,000 events for CK5, CK14 and CK18 (red), with an isotype control (green).

3.3.5 Cell Markers Representing other Functional Antigens

CD24, β-1 and α-6 integrins are transmembrane glycoproteins involved in cell adhesions which have all been identified in MaSC (Shackleton et al. 2006; Smith 2006). Murine mammary cells within the lin-CD29hiCD24low population were found able to reconstitute an entire functional mammary gland demonstrating at the highest level, what would constitute a true stem cell (Shackleton et al. 2006). The presence of ATP binding cassette G2 (ABCG2) protein which forms a channel on cell surface that is

involved in the trafficking of biological molecules across membranes, is also characteristic of many stem/ progenitor cells and also cancerous cell lines (Challen and Little 2006). Therefore, in addition to lineage specific markers, I also attempted to investigate protein expression of these markers which have been implicated in the identification of MaSC, namely CD24, β-1, α-6 integrins and ABCG2. Through flow cytometry, I found positive expression of the four proteins (Figure 3-15) in percentages as shown in Table 3-2 (page 79).

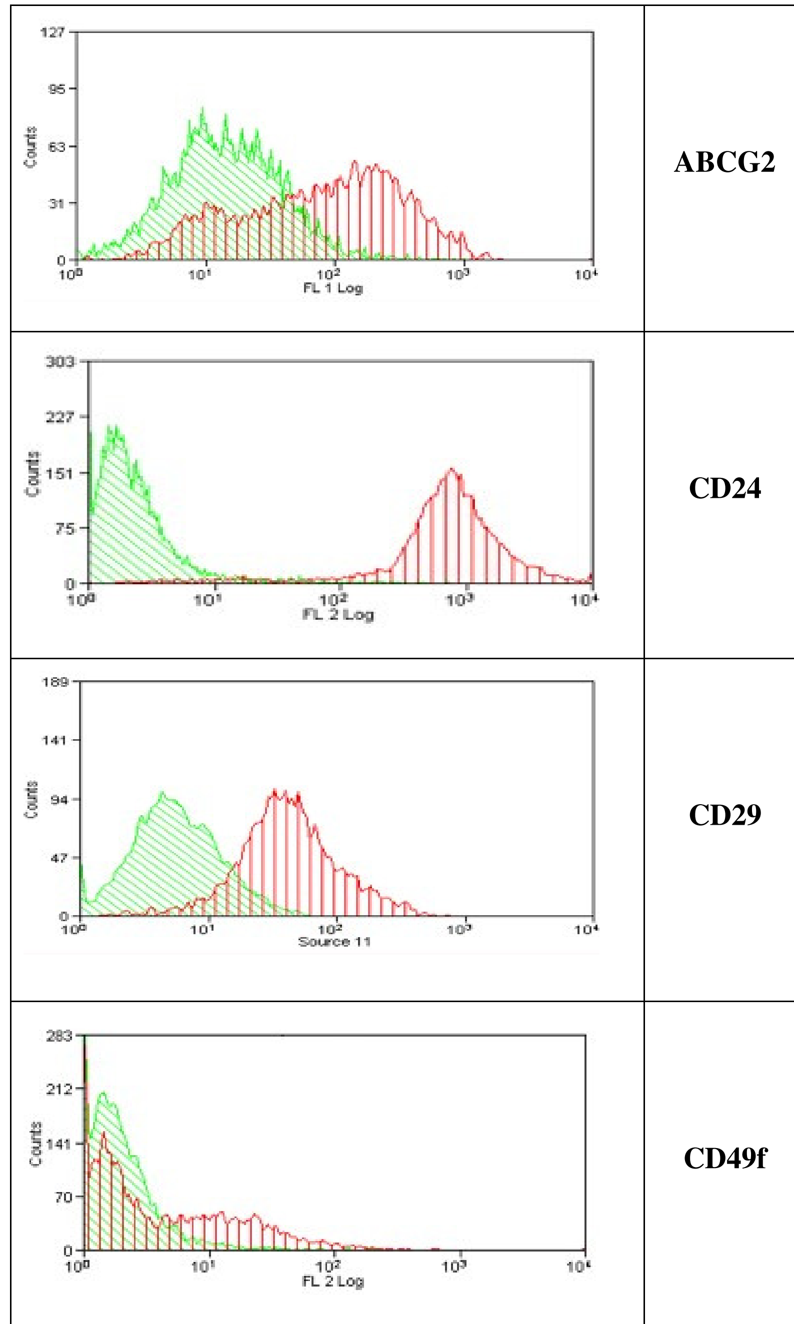


Figure 3-15 Staining for other functional proteins. Whole cell population directly isolated from HBM and subjected to flow cytometry, analyzing 20,000 events for ABCG2, CD24, CD29 and CD49f (red), with an appropriate isotype control (green).

3.4 Discussion

3.4.1 Summary of Results

Through the use of flow cytometry and RT-PCR, I have ascertained the positive expression of a large number of cell surface markers and intermediate filaments (IFs) within the cellular component of HBM. This includes stem/ progenitor markers of various lineages like CD133, ABCG2, nestin, Stro-1, CD24, β-1 and α-6 integrins and some of these markers have never been reported present in HBM or the cellular component of HBM.

3.4.2 Critical Assessment

My hypothesis was that adult stem/ progenitor cells are present in HBM. In order to determine the plausibility of this study, I investigated the cell surface markers and IFs on the cells of HBM using flow cytometry and RT-PCR to discover novel antigens that have never been investigated before.

Positive staining of stem/ progenitor markers of various lineages suggested the presence of uncharacterised cell types in the cellular component of HBM. The staining of ABCG2 further ascertains that potential stem/ progenitor cells are present. CD24 is a glycoprotein that is expressed on the surface of most B lymphocytes while β-1 and α-6 integrins interact with each other to form heterodimers. Interestingly, CD24, β-1 and α-6 integrins have also been implicated in MaSC (Shackleton et al. 2006; Stingl et al. 2006b). Shackleton et al have reported the isolation of mammary stem cell within the Lin-CD29hiCD24low population, with these cells able to reconstitute an entire

functional mammary gland demonstrating at the highest level, what would be a true stem cell, at least in the murine model (Shackleton et al. 2006). Stingl et al on the other hand also showed subsets of cells from reduction mammoplasties that expressed highest levels of CD29 or CD49f and CD24 (termed the ‘‘double positive’’ population). These double positive cells were found to be enriched for mammary stem/ progenitor cells by transplanting these cells at limiting dilution into cleared fat pads (Stingl et al. 2006b). I have shown the presence of all these antigens on the WCP of HBM (n=3) at substantial amounts.

I have also investigated the expression of a large number of IFs by flow cytometry, in view of their importance and specificity of their presence in cells. Intermediate filaments are one of the three major cytoskeletal protein filament systems of most vertebrate cells. They form networks that extend from the nucleus to the plasma membrane, attach to desmosomes, and interact with a variety of cell structures. They are essential in conferring tensile strength to the cells and play an indispensable role in determining the shape of cells (Markl and Schechter 1998). The different cytoplasmic IFs proteins are specifically expressed by different cell lineages: keratins in epithelia, vimentin in mesenchymal derived cells and tissues, desmin in muscle cells, glial fibrillary acidic proteins (GFAP) in astrocytes, and neurofilament proteins in neurons (Markl and Schechter 1998). Presence of keratins on mammary epithelium and the absence of desmin and vimentin in both epithelial and myoepithelial cells illustrate the specificity of expression of IFs (Franke et al. 1980). The presence of differentiated epithelial cells in HBM has been reported (Brooker 1980) and this correlates to my finding of CK14- and CK18-positive cells. The positive expression of CK5 and the

neural IFs once again, suggests the presence of a yet still uncharacterised cell population in HBM.

Epithelial and neural cells both descend from a common germ layer known as the ectoderm. The presence of nestin, which is a multipotent progenitor cell marker (Wiese et al. 2004), suggests the potential existence of a more primitive stem/ progenitor cell than mammary stem/ progenitor cells.

In conclusion, my results suggest the presence of novel cell type(s) in HBM. From the various antigens found present as well as the known established cell types in HBM, there is a high likelihood that this novel cell type(s) is primitive in nature, and likely to be either epithelial and/or haemopoietic.

4 In Vitro Expansion of Adherent Cells in Selective Medium

4.1 Introduction

In the previous chapter, I have demonstrated the presence of novel markers on the cellular content of HBM. In this part of my thesis, an attempt to identify the possible cell types will be made. By drawing from the anatomical location and the current knowledge of cell types established in HBM, I speculate that these cells are most likely epithelial cells. This, in combination with the established positive expression of mammary stem cell markers; CD24, CD29, CD49f and ABCG2 suggests that these cells are likely to be primitive epithelial cells.

The history of culture of epithelial cells in HBM dates back to 1972, where Beuhring and team introduced the derivation of epithelial cells from HBM to circumvent the difficulty in obtaining pure cultures of mammary epithelial cells. This method of obtaining epithelial cells from HBM and culturing them using Eagles's basal medium with 30% fetal calf serum, polymixin, antibiotic and antimycotic solved the problem of contaminating fibroblasts which inevitably accompanied the mammary epithelial cells during biopsies and tumour explants, which overgrows the epithelial cell types (Buehring 1972). Since then, there have been major improvements of the culture medium, which at its most initial form, is capable of achieving a confluent monolayer on a T_{75} flask over five days (Buehring 1972). Currently, the use of serum free defined culture medium has been formulated to grow MaSC as mammospheres, forming the gold standard for culture of MaSC (Dontu et al. 2003a).

In Chapter 3, I have documented the difficulty in staining cells from HBM due to the high level of cellular debris present, leading to non-specific binding of antibodies. This hindered their further characterisation. In this part of my thesis, I explored the possiblity

of expanding these putative primitive epithelial cell types. Enrichment of these cells-of-interests will enable more detailed characterisation of them and give some clue regarding their origin and behaviour. Taking into account the inclination of epithelial cells to be adhesion-dependent, I first explored cell expansion through two-dimensional monolayer cultures. I followed this up with a three-dimensional matrigel culture to simulate the in-vivo conditions within the mammary gland.

4.2 Two-dimensional Monolayer Culture

Cells isolated from gravitational centrifugation were plated into 24-well plates at a density of 10^5 cells/ ml of medium. This was left overnight in the incubator for the epithelial cells to adhere using a medium that selectively aided the growth of epithelial cells. The wells took 7 to 30 days to reach confluency with medium changes every two to three days. The non-adherent cells were removed by the series of medium changes. Confluent monolayers as shown in (d) of Figure 4-1 were then fixed for immunocytochemistry work.

4.2.1 Growth Kinetics

There was great variation in the growth potential of the isolated cells. Cells of sample shown in Figure 4-1 adhered within four days and reached confluency in approximately 18.0±4.0 days (Figure 4-2).

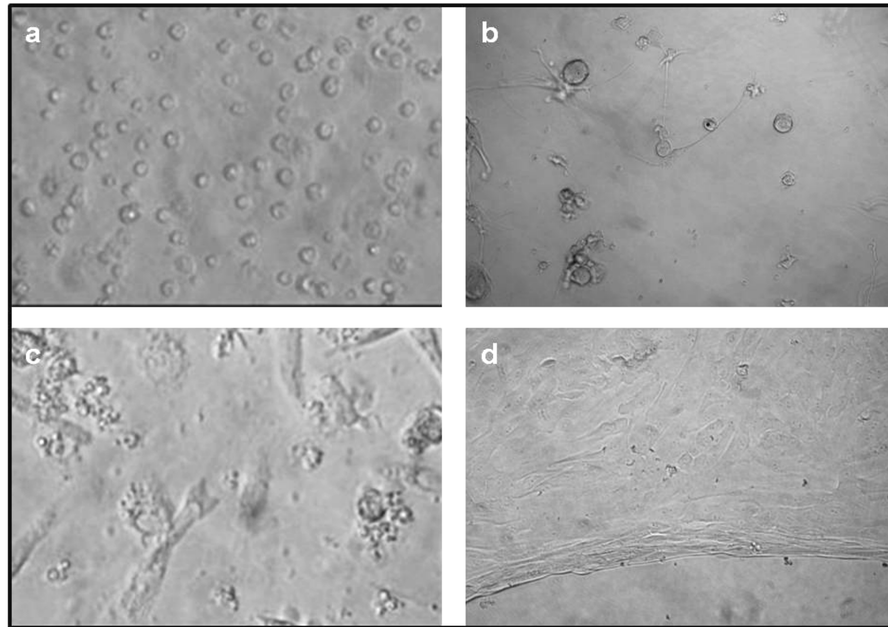


Figure 4-1 Two-dimensional monolayer of cultured epithelial cells. Cells were left to adhere for a day (a), after which attachment began (b) and dividing proceeds beyond day 4 (c) to day 18 (d) where a confluent monolayer was observed which is then fixed for immunocytochemistry work. Magnification of 20X (a, b and d) and 40X (c).

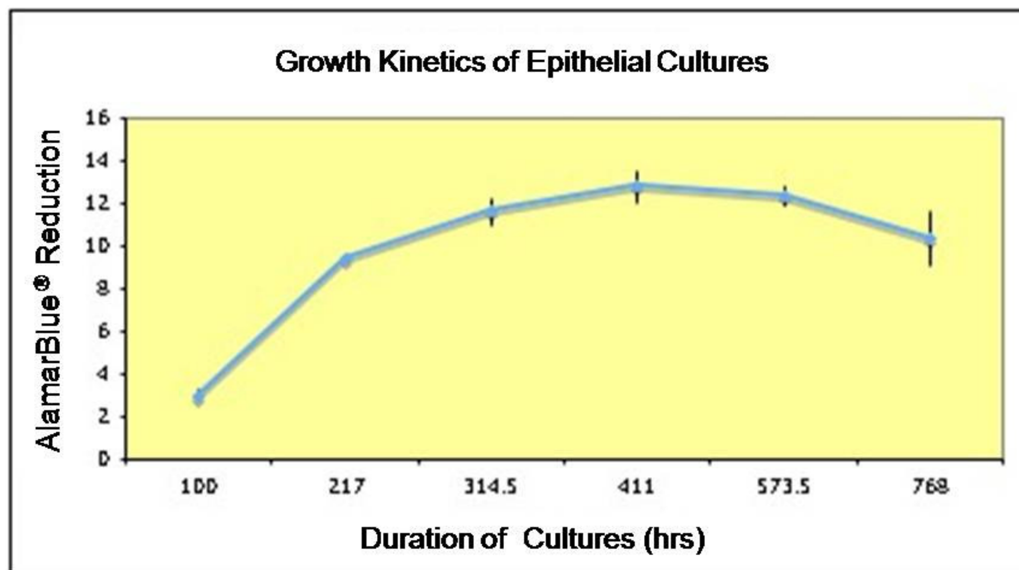


Figure 4-2 Metabolic activity of cells grown in 2-D cultures using AlamarBlue®. Standard error means are denoted by the black bars.

This growth trend was confirmed by the use of a metabolic assay (AlamarBlue®), where metabolic activity rises gradually until day 17 where it plateaus, coinciding with confluence of the culture (n=3) (Figure 4-2).

4.2.2 Characterisation of Intermediate Filaments in Adherent Cells

4.2.2.1 Detection of RNA by RT-PCR

I performed RT-PCR for the immature CK5, and more mature lineage specific CK14 and CK18. Identification of these IFs would demonstrate mammary epithelial cells of different maturity (Franke et al. 1979; Taylor-Papadimitriou and Lane 1987). I also attempted to explore expression of nestin in the cultured cells as a follow-up to the presence of nestin-positive cells in uncultured WCP of HBM. Semi-quantitative analysis of the RT-PCR was done to investigate how the expressions of the IFs change when the cells are placed into culture. Comparatively, I found a similar expression of CK18 between culture-expanded cells and uncultured WCP, but a much lower expression of CK5 and CK14 in the uncultured WCP. This is demonstrated in Figure 4-3 where CK5 and CK14 expression was below the detection threshold of RT-PCR, while CK18 was readily detected in cells directly isolated from milk. This suggests that epithelial cells are sustained in the adherent monolayer. There seemed to be an increase in the mammary progenitor population as indicated by the increase in mRNA level of CK5. Consistent with my earlier findings [Section 3.3.3], I also found the presence of nestin in the cultured cells (Figure 4-3).

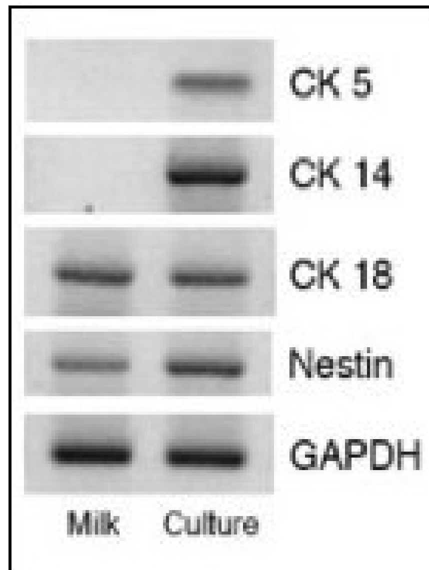


Figure 4-3 RT-PCR of intermediate filaments. Expression of CK5 and CK14 were up-regulated in cultured cells. Similar expression of CK18, nestin and GAPDH in the cells isolated from HBM and culture were observed.

4.2.2.2 Detection of Protein by Immunocytochemistry

4.2.2.2.1 Single staining with horseradish peroxidase

Positive staining for CK5, 14, 18, 19 and nestin were ascertained by comparing with the negative controls and the percentages of stained cells were counted. I noted 50.5±7.0% positive staining for CK5 (n=21), 22.3±3.9% positive staining for CK14 (n=24), 42.0±7.4% staining for CK18 (n=16) and 32.4±6.8% staining for CK19 (n=17). In terms of nestin staining, I observed 14.2±3.0% of nestin-positive cells in the cultures (n=17). In line with other reports, we found positive staining for epithelial specific CK18 and CK14 cells. From the counting of stained cells and as shown in Fig 4-4 and 4-5, we observed not only a higher percentage of cells stained with CK18 and also at a much greater intensity compared to CK14. Interestingly, I observed a population of cells which co-expressed both nestin and CK5 in cells cultured from HBM (Figure 4-6).

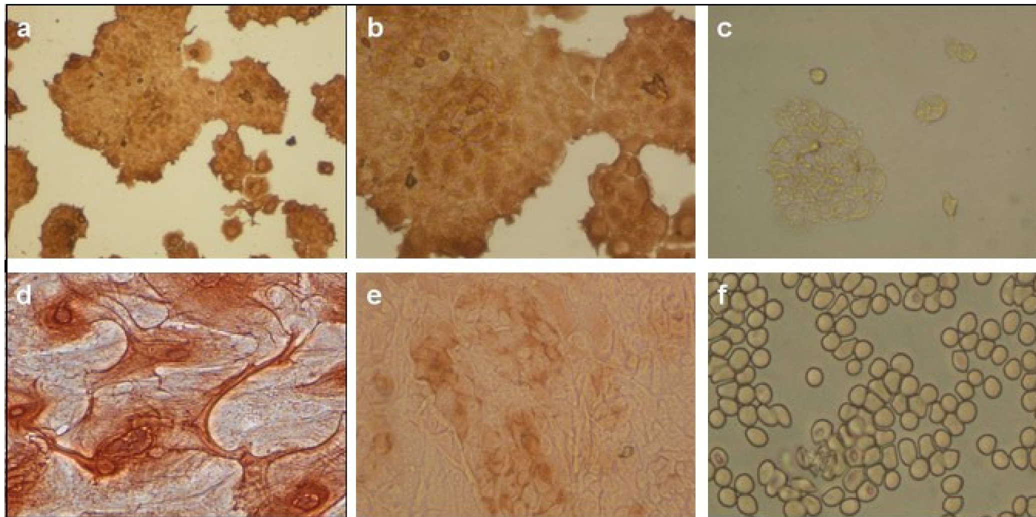


Figure 4-4 Immunocytochemistry for CK18. Homogenous positive stainings of cultured MCF-7 cells were observed (a-c) (positive control, c without primary antibody). In the cultured cells, heterogeneous staining indicates differential expression of CK18 (d, e). Erythrocytes serve as negative control (f). Magnification of 4X (a, b), 20X (c,e,f) and 40X (d).

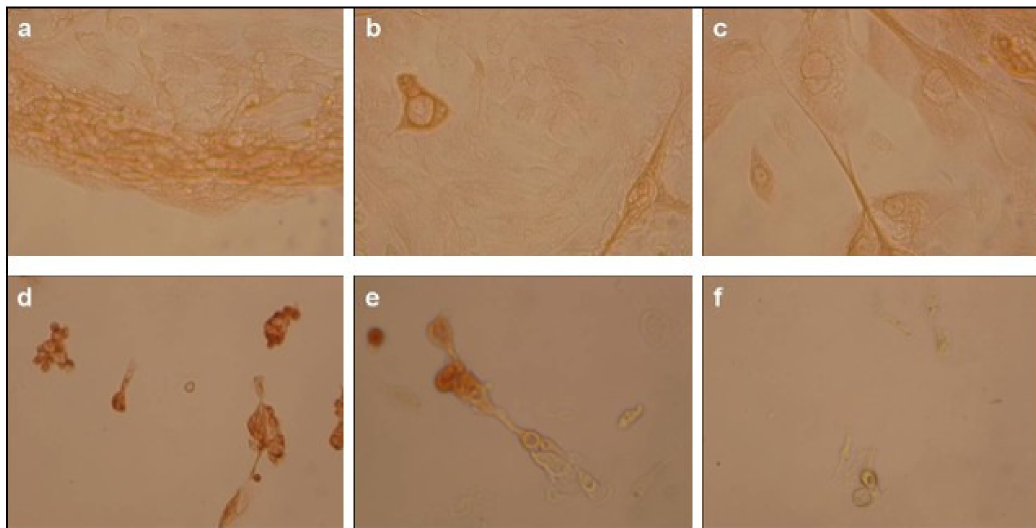


Figure 4-5 Immunocytochemistry for CK14. Lighter stainings were observed for CK14, which stained 22.3±3.9% of the cultured cells (a-e). Positive staining for CK14 was done by comparing with the internal negative control without 1º antibody (f). Magnification of 20X (a, d, e, f) and 40X (b, c).

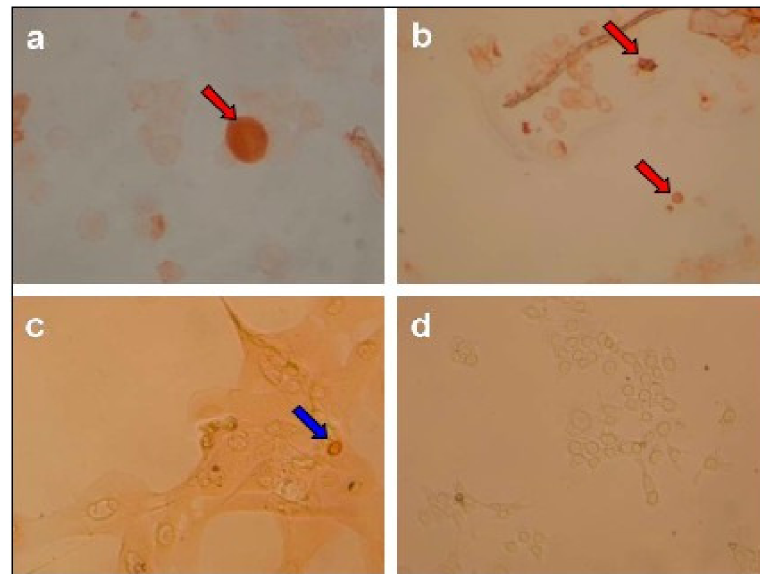


Figure 4-6 Immunocytochemistry for CK5 and nestin. Nestin staining (red arrows) were observed in 14.2±3.0% (n=17) of the cells (a, b). 50.5±7.0% (n=21) of the cultured cells stained for CK5 (blue arrow) (c). Positive stainings were determined by comparing with internal negative controls without primary antibody (d). Magnification of 40X (a) and 20X (b, c, d).

CK5 and CK14 were not expressed in HBM by means of RT-PCR but shown to be present in culture-expanded cells from HBM. Hence, I segregated the staining profile of the above IFs based on the number of the days the cells were cultured with the intention of looking for trends of change in expression of the 5 IFs. I segregrated the staining profile into three different culture periods, between 7 and 10 days (denoted as EM), 11 and 20 days (denoted as MM) and beyond 21 days (denoted as LM). These findings were tabulated as shown in Table 4-1. There was statistical difference found only in CK18 (p-value = 0.04, ANOVA, Kruskal-Wallis test).

	EM	MM	LM
CK5	53.0±11.3	59.1±15.9	44.1±11.6
CK14	21.2±3.7	18.4±7.9	26.0±6.1
CK18	22.6±2.7	74.0±11.1*	22.4±9.2
CK19	24.4±5.8	28.4±8.4	32.0±17.7
Nestin	15.8±5.6	14.2±4.5	6.73±2.1

Table 4-1: **Expression of IFs based on duration of monolayer cultures.**

4.2.2.2.2 Dual staining with fluorescent tags

In order to delineate the identity of these culture-expanded cells, IF proteins were stained using double labelling of nestin with CK5, 14, 18 and 19. There is as yet no definitive marker to demonstrate identity of MaSC. However, there have been reports on the use of dual staining to suggest identity as a mammary stem/ progenitor cell. Previous publications have shown that the myoepithelial and luminal epithelial cells were derived from the same progenitors. These progenitors co-express CK14 and CK18 and lose one of the two markers when differentiate into one specific terminal epithelial cells (Pechoux et al. 1999). CK5, 14 and 19 have also been proposed as mammary stem/ progenitor markers, though not definitively as CK14 is also a marker of myoepithelial cell and CK19 being a marker of luminal epithelial cells, like CK18 (Bocker et al. 2002; Boecker and Buerger 2003). In congruence with the earlier findings on immunocytochemical staining of single antigens through horseradish peroxidase, I observed positive staining for all 5 IFs (Figure 4-7, 4-8). Using fluorophore reporter tags, I observed 47.2±7.6% of cells being CK5-positive, 17.8±3.7%, 28.3±6.5 and 23.6±5.1% being CK14-, 18- and 19-positive respectively. Expression of nestin was

present in 16.0±2.6% of cells counted. Although some differences in levels of positivity were observed particularly for CK18 and CK19 between detecting with horseradish peroxidase and fluorophore reporter, these differences turned out insignificant (p-value > 0.2), suggesting no differences between the staining methods. Nestin, a multipotent stem/ progenitor cell marker was found present in the cultured cells at both mRNA and protein level (Figure 4-3, 4-6 and 4-8). Nestin was co-expressed with CK19 (24.4±3.6%), CK5 (11.4±4.2%), CK14 (3.7±1.5%) and CK18 (3.3±2.7%) in decreasing frequencies (Figure 4-8).

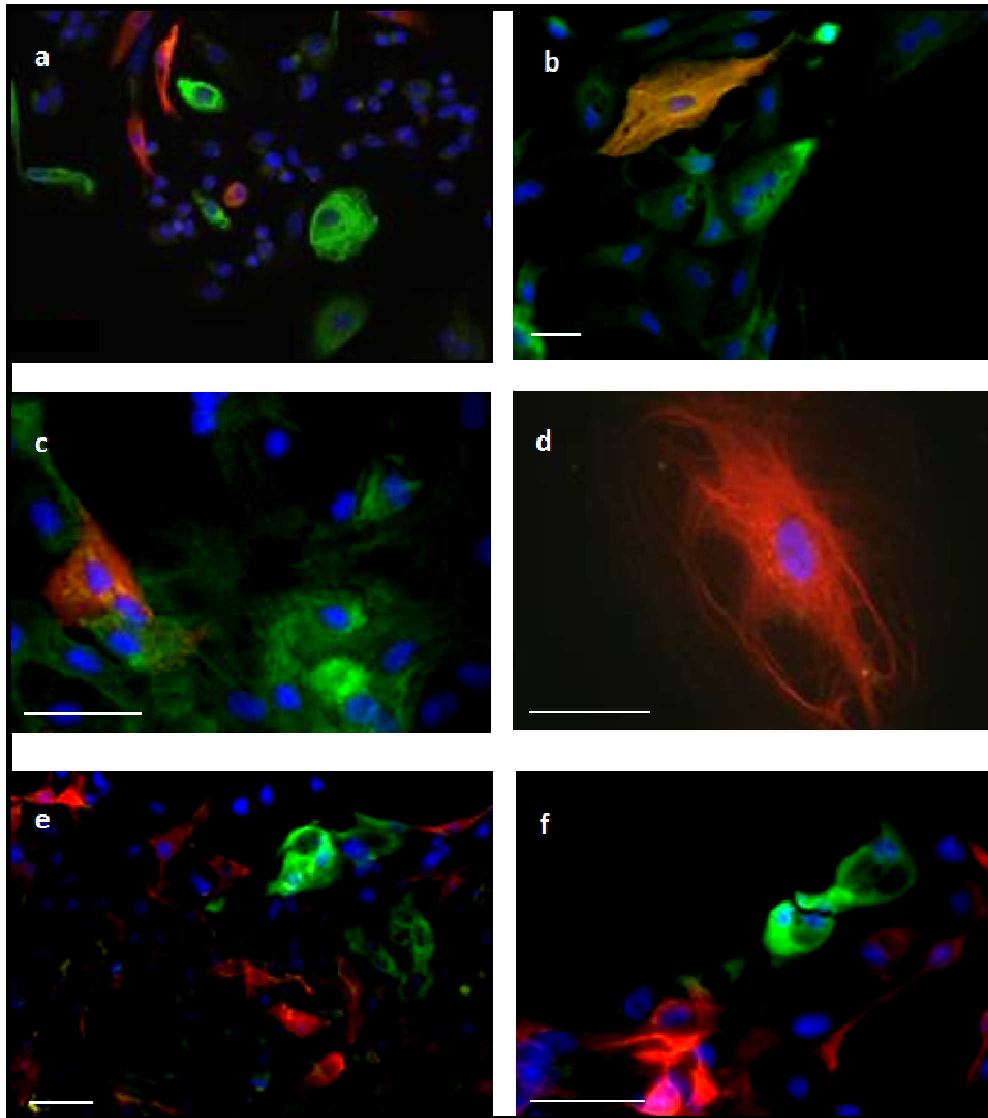


Figure 4-7 Expression of cytokeratin markers in cells cultured from HBM. Fluorescent immunocytochemistry imaging of dual staining for CK14 (red), and CK18 (a-b), CK5 (c-d) or CK19 (e-f) (green). CK18+ luminal epithelial cells were round and larger when compared to the CK14+ myoepithelial cells, which appears spindle-shaped (a). Scale: 50µm.

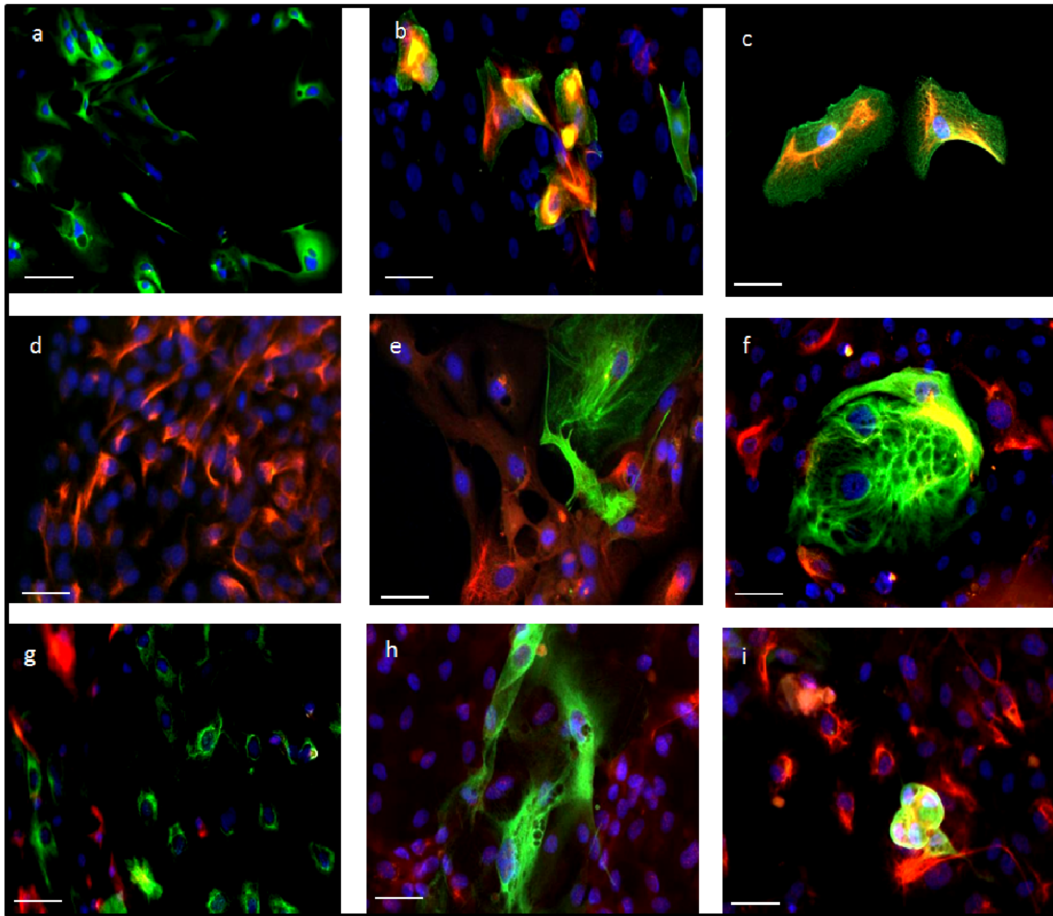


Figure 4-8 Expression of the multipotent marker, nestin with cytokeratins. Dual fluorescent staining for nestin (red) with CK 5 (a-c), CK14 (d-e), CK 18 (f-g) or CK 19 (h-i) (green) in cells cultured from HBM. Scale: 50µm.

4.3 Three- dimensional Culture on Matrigel

Three-dimensional culture of the epithelial cells was carried out to simulate a physiological relevant microenvironment, to allow stromal-epithelial interactions, mimicking the in vivo development of mammary gland. Matrigel, generated from Engelbreth-Holm-Swarm sarcoma was chosen as it contains not only basement membrane components (collagens, laminin, and proteoglycans) but also matrix degrading enzymes, their inhibitors and growth factors. In addition, there have been several reports of positive cultures of luminal, myoepithelial and precursor cells in

matrigel forming acinus-like structures, solid colonies and terminal ductal lobular units respectively (Petersen et al. 1992; Gudjonsson et al. 2002b).

4.3.1 Growth Kinetics

Cells grown as monolayers were trypsinised and cultured in matrigel-coated plates with the same medium. Matrigel is believed to induce differentiation of the cells, as has been reported by Dontu's group using mammary stem/ progenitor cells isolated from reduction mammoplasties (Dontu et al. 2003a). Cells plated on matrigel-coated plates were cultured for 16±2days (n=40) before being used for immunocytochemistry.

4.3.2 Characterisation of Intermediate Filaments

I performed immunocytochemistry on the cells grown in matrigel coated plates and observed 52.4±6.2% (n =10) of the cells being nestin-positive. This is a steep increase of nestin's expression from the 34.2 ± 4.3% (n= 21) in cells grown as monolayers. A mean percentage of 50.5±13.3% (n=8) cells in matrigel cultures were CK5-positive. 46.2±11.7% (n=9), 33.4±14.4% (n=5) and 34.6±9.6% (n=8) of the cells grown in matrigel were found CK14-, CK18- and CK19-positive respectively.

There were no differences in the proportion of positively-stained cells for each IF between two and three dimensional cultures as shown in Table 4-2 except for nestin.

	2D	**3D**	**p-value**
CK5	58.5±6.5	50.4±13.3	0.56
CK14	34.6±5.3	46.2±11.7	0.31
CK18	48.4±7.6	33.4±14.4	0.24
CK19	43.8±6.4	34.6±9.6	0.44
Nestin	34.2±4.2	52.4±6.2	0.02

Table 4-2: **Expression of IFs by immunocytochemistry based on types of cultures.**

4.4 Discussion

4.4.1 Summary of Results

From the growth kinetics, it is evident that epithelial cells from HBM have a wide range of proliferation profile, and that they are able to proliferate in both two and three-dimensional culture systems.

I have noted the presence of nestin and CK18 mRNA on cells directly derived from HBM (Figure 4-3). Interestingly, these two IFs as well as CK5 and CK14 were expressed when cells from HBM were cultured. From the cells directly isolated from HBM, using semi-quantitative RT-PCR, I observed a similar amount of staining for CK18 whereas after culturing these cells as monolayers, I observed the appearance of CK5 and CK14.

From the co-staining experiments, I observed that heterogeneous cultures of nestin, CK5, CK14, CK18 and CK19 were common and that nestin had the highest proportion of co-staining with CK19, followed by CK5 , CK14 and lastly, CK18 (Figure 4-8).

4.4.2 Critical Assessment

4.4.2.1 Growth Kinetics

I was able to grow the cells isolated from HBM both in two and three-dimensional cultures although not in a manner I have anticipated. Various groups have grown cell lines on three-dimensional in vitro culture models and observed the formation of structures resembling those in the mammary glands (Bae et al. 1993; Krause et al. 2008). I sought to investigate the formation of acinar structures using cells from HBM with 3D matrigel cultures but did not observe any formation of complex structures similar to acini.

4.4.2.2 Pattern of Staining

4.4.2.2.1 Two-dimensional monolayer cultures

CK5 and CK14 were not detected in the WCP of HBM but appeared de novo in the culture expanded cells (Figure 4-3 and 4-7). This could be explained in three ways. The first explanation is that CK5- and CK 14-positive cells originated from either the nestin-positive or CK18-positive cells or even both of them. Nestin is a well-characterised marker of multipotent progenitor cells and is closely associated with stem cell populations in bone marrow (Kabos et al. 2002), neural (Lendahl et al. 1990; Dahlstrand et al. 1992), pancreatic (Zulewski et al. 2001) and epithelial (Toma et al. 2001) tissues. This in combination with the presence of nestin+/ CK5-, nestin+/ CK5+,

and nestin^{-}/ CK5+ cell types (Figure 4-8) suggest that nestin+ cells found in HBM might be the precursor cell type.

Alternatively with the semi-quantitative RT-PCR results taken into consideration, the other possible explanation for my observations would be that the culture media selectively enabled the CK5- and CK14-positive cells to expand more than the CK18-positive cells. This would explain why these cells although present in low proportions in WCP of HBM, which were insufficient to give a positive signal during RT-PCR, manage to give positive signals for CK5 and CK14 after being cultured.

The third explanation for my observation would that the culture media had brought about dedifferentiation of the CK18-positive cells into the CK5- and CK14-positive cells. In addition to explaining the appearance of bands for CK5 and CK14 in Fig. 4-3, this explanation would also account for the larger band of nestin observed in Fig. 4-3.

4.4.2.2.2 Three-dimensional matrigel cultures

The higher expression of nestin exhibited by cells cultured in matrigel-coated plates suggests a possible dedifferentiation of the epithelial cells, which contrasted with Dontu et al's findings that matrigel induces differentiation into terminal structures (Petersen et al. 1992; Gudjonsson et al. 2002b; Dontu et al. 2003a). The lack of statistical significance in the cytokeratin staining however suggests that my postulation of dedifferentiation, based on the expression of nestin is unsubstantiated.

4.4.2.3 Hierachy of Adherent Cells in HBM

Based on the IFs found on the cells in HBM as well as established information of these IFs, I postulate a hierarchy of cells within the lobulo-alveoli structure (Figure 4-9). I have shown that expressed HBM is a novel source of nestin+ putative stem cells. The capability of these nestin-positive cells in HBM to differentiate into cell types of other lineages should be explored since reports of nestin-positive stem cells transdifferentiating into other cell types are numerous (Zulewski et al. 2001; Kabos et al. 2002; Amoh et al. 2005).

Due to their partial co-expression with CK5 and anatomical origins, I postulate that they are mammary stem/ progenitors, which have been shown present. However, we will not be able to isolate the primitive cells-of-interest with these IFs, as they are intracellular and cells will have to be permeabilised, thereby destroying their plasma membrane. In my following chapters, I hence decide to use two surface markers, known to exist on stem cells, to isolate these primitive cells-of-interest to carry out further characterisation in order to ascertain their stemness.

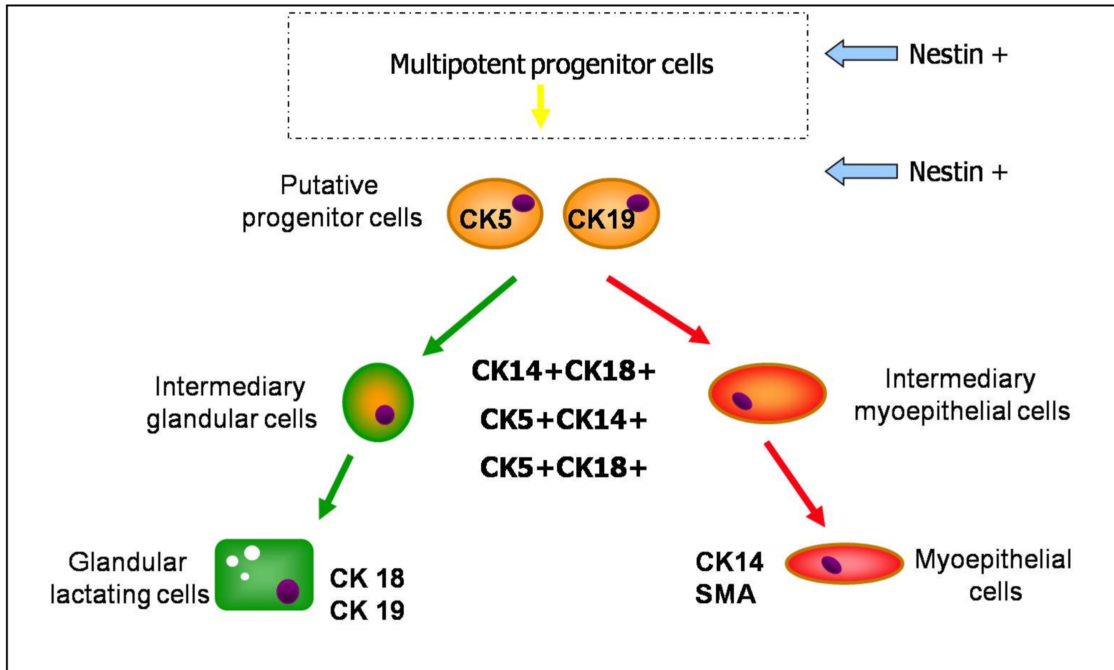


Figure 4-9 Flow chart for hierarchy of stem/ progenitor cells of mammary lineage.

5 Isolation of Stem/ Progenitor Cells in Expressed Human Breast Milk by Hoechst Dye Exclusion

5.1 Introduction

In the previous two chapters, I have firstly established the presence of yet-unknown cells in HBM and secondly, identified the presence of nestin-positive cells in HBM. I postulate that these nestin-positive cells are the most primitive cell type present in HBM. As it would not be possible to prospectively isolate them using nestin which is an intermediate filament, I decide to look for appropriate cell surface proteins that are commonly found on stem/ progenitor cells to attempt the isolation of these cells.

The use of cell surface markers to isolate cells is ideal as we would then be able to isolate viable stem/ progenitor cells. In fact, there are several cell surface markers that are already commonly in use to isolate primitive cells. One of the newer markers found by Goodell and team in 1996 is cell membrane channel which seem to be present on haemopoietic stem cells (Goodell et al. 1996).

ABCG2 proteins function as channels on cell membranes to allow active efflux of substances, including toxins. The functionality of these channels can be demonstrated in vitro by the cells' ability to efflux Hoechst dye. The group of cells capable of active efflux of Hoechst dye are termed the side-population (SP). Goodell et al. found an enrichment of haemopoietic stem cells in the SP of bone marrow (Goodell et al. 1996). Since then, SP has been reported in many stem cell populations, including mammary stem/ progenitor cells (Dontu et al. 2003a; Challen and Little 2006). Islam et al also found the co-localisation of nestin and the ABCG2 protein, on the same cells of many neurospheres (Islam et al. 2005) while Hayashi and team have made use of FACS and cell surface markers integrin α6 and CD71 to enrich for postulated corneal stem/ progenitor cells which he showed to have higher clonogenic capacity, a larger

proportion of non-dividing cells, and also express a higher level of ABCG2, a marker for stem/ progenitor cells (Hayashi et al. 2008).

Hence in this chapter, I sought to identify viable cells-of-interest by this property of Hoechst dye exclusion in HBM, with the aim of isolating and characterising the primitive cell types.

5.2 Occurrence of Side-population

In cells directly isolated from HBM, I observed a side population (SP) that excluded Hoechst 33342 dye (Figure 5-1a). This group of cells was absent in the controls where Verapamil was used to block the membrane transporters (Figure 5-1b). I repeated Hoechst dye exclusion experiments on 47 milk samples (from 7 different mothers at various intervals) and found a distinct SP in 40 of the 47 samples (85.1%). A mean of 2.1±0.3% of the entire cellular component was found within the SP. There was no significant correlation between samples from different donors as well as between samples collected from the same donor over a period of six months (Figure 5-2). This indicated that SP exists in HBM at a relatively constant percentage throughout duration of lactation.

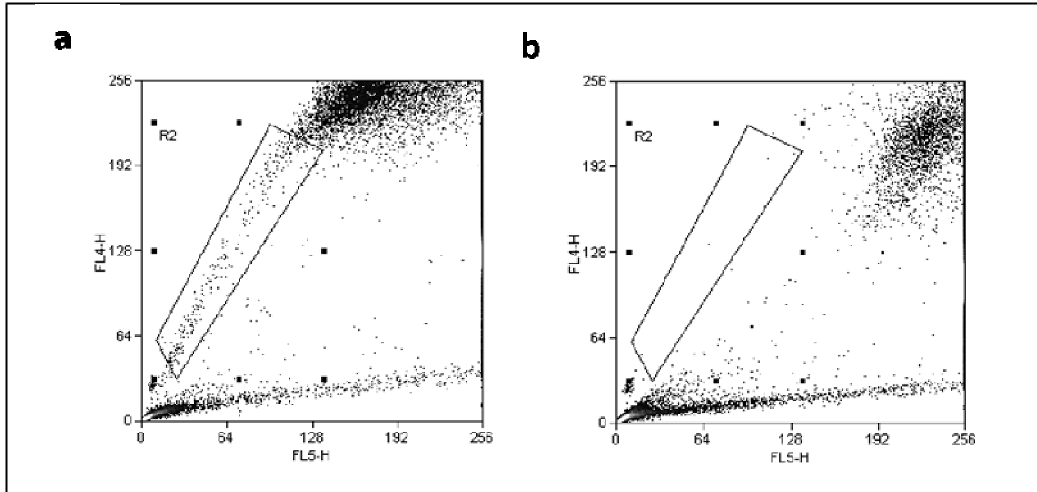


Figure 5-1 Hoechst 33342 exclusion by SP in HBM. SP can be identified in approximately 2% of WCP (gated R2), while the majority of cells (ungated) stained intensely with Hoechst (a). Verapamil block the ABCG2 channels and serve as a negative control (b).

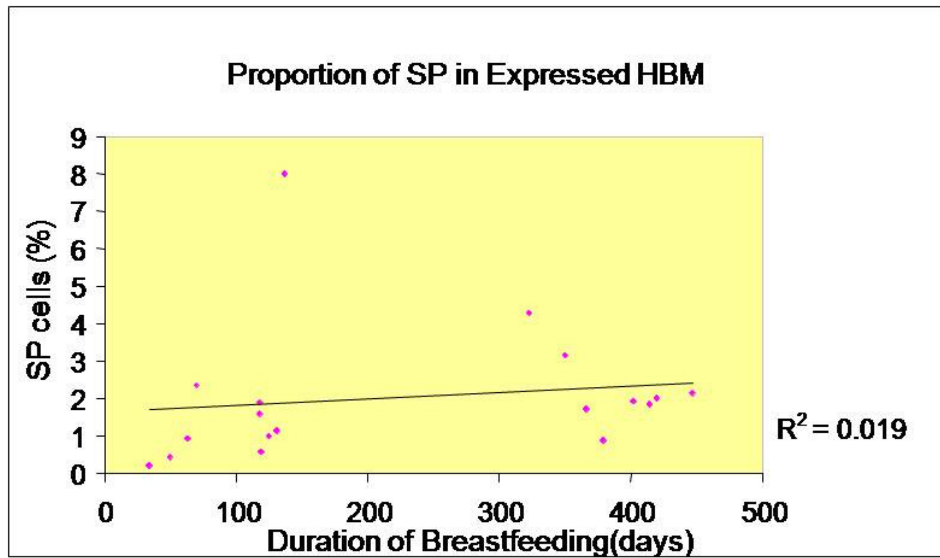


Figure 5-2 Correlation analysis of SP with duration of breastfeeding. Scatterplot showed the lack of correlation between the percentage of SP cells and the duration of breastfeeding.

5.2.1 Controls

Unless stimulated by granulocyte stimulating factor, adult peripheral blood does not usually contain stem/ progenitor cells and therefore it would be a good external negative control, while mononuclear cells from umbilical cord blood were used as positive controls to establish the method of Hoechst dye exclusion. Mononuclear cells from peripheral blood were incubated with Hoechst 33342 dye and no SP was observed (Figure 5-3a-b), while a SP was evident in the positive control (Figure 5-3c-d).

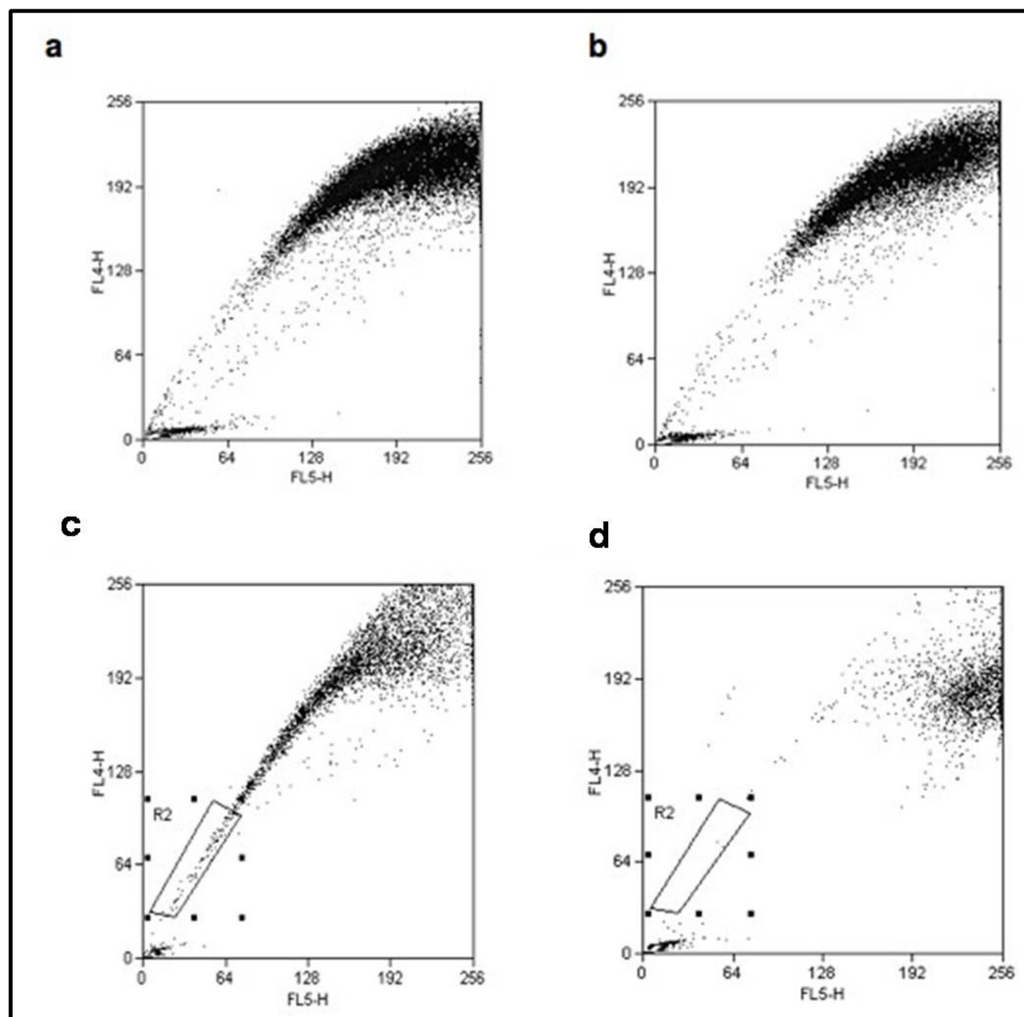


Figure 5-3 Hoechst dye exclusion of controls. Incubation with Hoechst alone (a) was no different from the Verapamil control (b) in mononuclear cells of peripheral blood, indicating absence of SP whereas a clear region of SP cells was present in the mononuclear cells from umbilical cord blood (c) which is absent when cells were incubated in the presence of Verapamil (d).

5.3 Characterisation of Side-population

Cells in the SP and non-SP were collected separately and expression of various proteins investigated by immunofluorescence and flow cytometry ($n \geq 3$). The sorted cells were stained for nestin and CK18 using immunofluorescence. SP cells were predominantly nestin-positive (89.7±4.7%), compared to negligible expression in non-SP cells (0.67±0.54%, p-value < 0.001) (Figure 5-4b). In contrast, more non-SP than SP cells stained positive for the mature epithelial marker CK18 (39.4±9.8 vs 5.3±2.0%, p-value = 0.026) (Figure 5-4c compared to 5-4d).

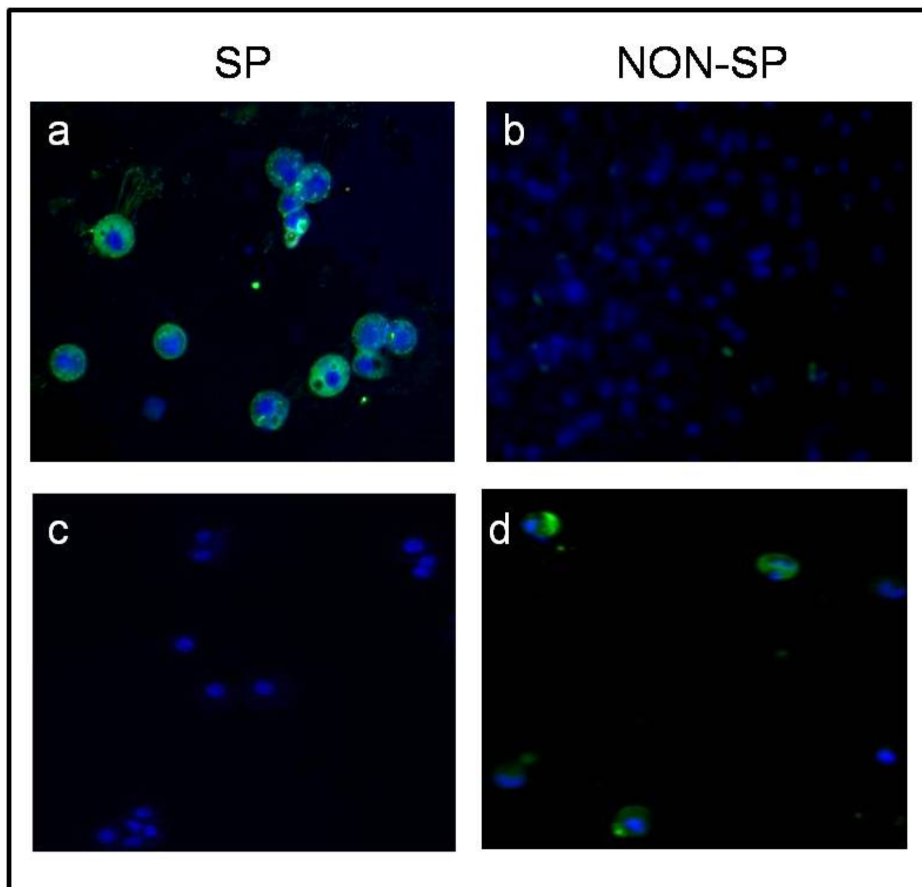


Figure 5-4 Immunocytochemical staining for nestin and CK18. Nestin staining of SP demonstrated predominantly positive cells (green cytoplasmic staining) (a) while largely negative in non-SP cells (b). On the other hand, CK18 staining was found predominantly in non-SP cells (c-d). Nucleus counterstained in DAPI. Magnification of 20X.

The expression of ABCG2, CD45, CK5 and CK14 were investigated with flow cytometry (Figure 5-5). The low expression of ABCG2 in both populations suggested the presence of other protein channels responsible in bringing about the efflux of Hoechst dye. Presence of CD45 was observed in both SP and non-SP cellular fractions (5.7±2.2% vs 12.7±4.6%, Figure 5-5a). This illustrates the contribution of haemopoietic cells to both populations. A smaller proportion of SP cells was found CD45-positive as compared to non-SP cells, although not reaching statistical significance. Expression of the CK5 and CK14 were comparable between the SP and non-SP cells (9.4±4.4% vs 12.1±5.9% and 15.6.7±5.4% vs 10.4±4.2% (Figure 5-5b).

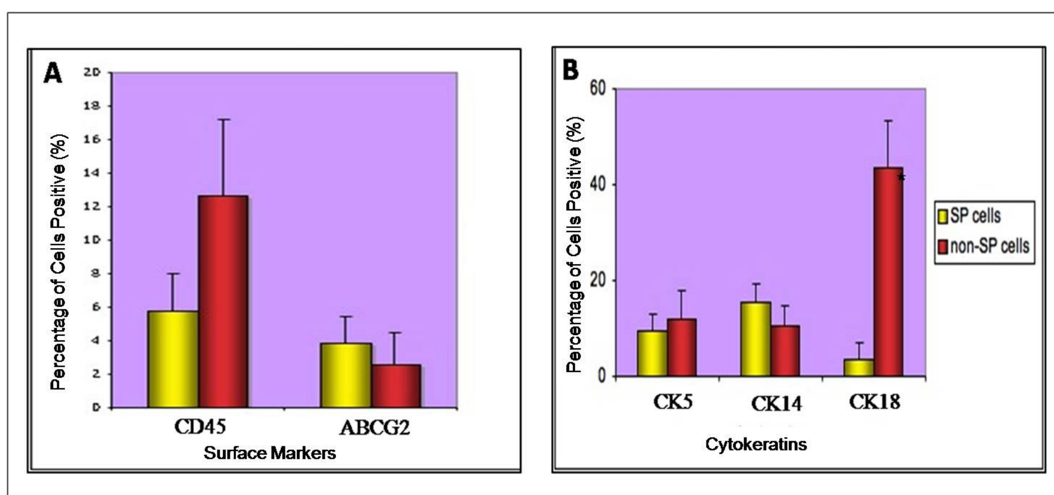


Figure 5-5 Flow Cytometry for CD45, ABCG2, CK5, CK14 and CK18. Comparison of expression of CD45 and ABCG2 (a), of CK5, CK14 and CK18 (b) between the SP and non-SP.

In order to further delineate the two populations, I stained flow-sorted SP and non-SP for the expression of other epithelial and non-epithelial markers (Table 5-1). EPCAM, which is known to be expressed by bi-potent mammary progenitor cells, which give rise to EPCAM-positive luminal progenitors and EPCAM-negative myoepithelial

progenitors (Stingl et al. 2001; Eirew et al. 2008), was found to be enriched in the SP fraction (50.6±8.6% vs 18.1±6.0%, p-value = 0.02). We found a small increase in CD31-positive cells in the SP (1.9±.2% vs. 0.7±0.3%, p-value = 0.04) over the non-SP fraction, which may indicate the presence of haemopoietic and/ or endothelial cells, which are known to express CD31 (Watt et al. 1995), within the SP.

Taken together, the enrichment of cells with primitive markers EPCAM and nestin and the lower expression of the mature epithelial marker CK18 in the SP cellular fraction of WCP suggest the presence of MaSC within uncultured WCP in HBM.

Properties	Antigen	Expression on SP	Expression on NSP	*P*-value
Stem/ Progenitor	ABCG2	3.9±1.6	2.5±1.9	0.78
	CD133	6.1±1.9	3.9±2.0	0.20
Mammary Stem Cell	CD44	5.9±2.4	23.3±10.0	0.25
	CD24	47.2±19.1	37.5±17.7	0.42
	CD29	0.3±0.2	2.7±1.9	0.36
	CK5	9.4±4.4	12.0±6.0	0.79
Mammary Epithelial	CD10	3.9±2.3	1.7±0.7	0.53
	EPCAM	50.6±8.6	18.1±6.0	0.02*
Non-epithelial	CD45	5.7±2.3	12.7±4.6	0.25
	Lin	37.8±17.8	5.7±1.5	0.29
	CD105	27.9±9.9	6.5±4.4	0.32
	CD31	1.9±0.2	0.7±0.3	0.04*

Table 5-1: **Antigens expressed on the SP and NSP in HBM.**

5.4 Mammosphere Culture of Selected Populations

5.4.1 In Vitro Expansion of Side-population

Expansion of these rare cells is important in an attempt to increase cell numbers to work with. There is also a need to prove clonogenicity and self-renewal ability of these cells to identify them as stem cells (Lajtha 1979; Humphries et al. 1981). Prior to this, I devised a series of positive controls as will be discussed in Section 5.4.1.1.

5.4.1.1 Positive Controls for Mammosphere Culture

To prepare for mammosphere cultures for my cells-of-interest, there is a need to ascertain the functionality of my Hoechst dye exclusion protocol as well as mammosphere medium.

5.4.1.1.1 Culture of neurospheres

Using neurospheres as a working system for the culture of spheroids in vitro, I embarked on the generation of neurospheres derived from fetal brain tissues. Single cells derived from eight various regions of the brain were plated into neurosphere medium to grow. Neurospheres as shown in Figure 5-6 were then observed after two to four weeks of culture.

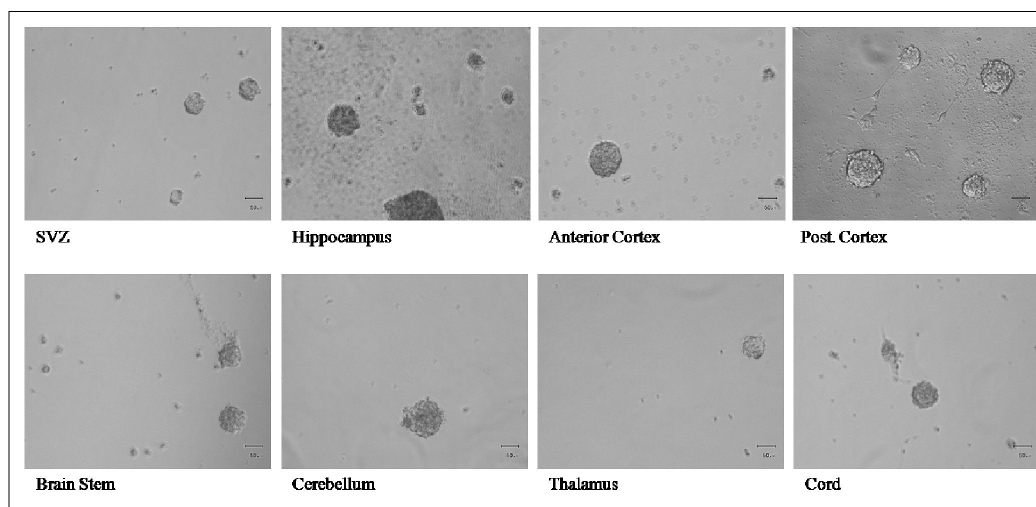


Figure 5-6 Free-floating neurospheres cultured from fetal cells. Spheres derived from the various regions were morphologically similar. Magnification of 10X.

Overall, spheres were observed growing in 5 out of 5 samples and across each sample, spheres generally grow from all regions. Morphologically, the spheres were identified by their phase-bright appearance and regular cell membranes and microscopically, there

is little difference in the physical appearance of the neurospheres derived from the different regions of the second trimester fetal brain (Figure 5-6). The number of spheres larger than 50µm in diameter were counted and a low efficiency of lesser than 0.09% across the second trimester samples. From the fetus of the smallest gestational age (14+6weeks), we observed the lowest neurosphere initiating assay in the hippocampus (0.002±0.00004%) and the highest in the brain stem (0.0074±0.009%) and from the fetus of the oldest gestational age (23+1weeks), we observed once again, the lowest neurosphere initiating assay in the hippocampus (0.006±0.00004%). However, the anterior cerebrum was found to have the highest efficiency in neurosphere generation this time round (0.066% ± 0.0162%) (Figure 5-7). From the graphical representation in Fig. 5-7, an increasing trend for neurospheres' initiating ability was observed between 14weeks and 23weeks, suggesting that the largest number of neural stem cells in second trimester fetus is present in the later phase of second trimester, specifically 20weeks in the case of subventricular zone (SVZ) and hippocampus and 23+1weeks for the other five regions of the fetal brain.

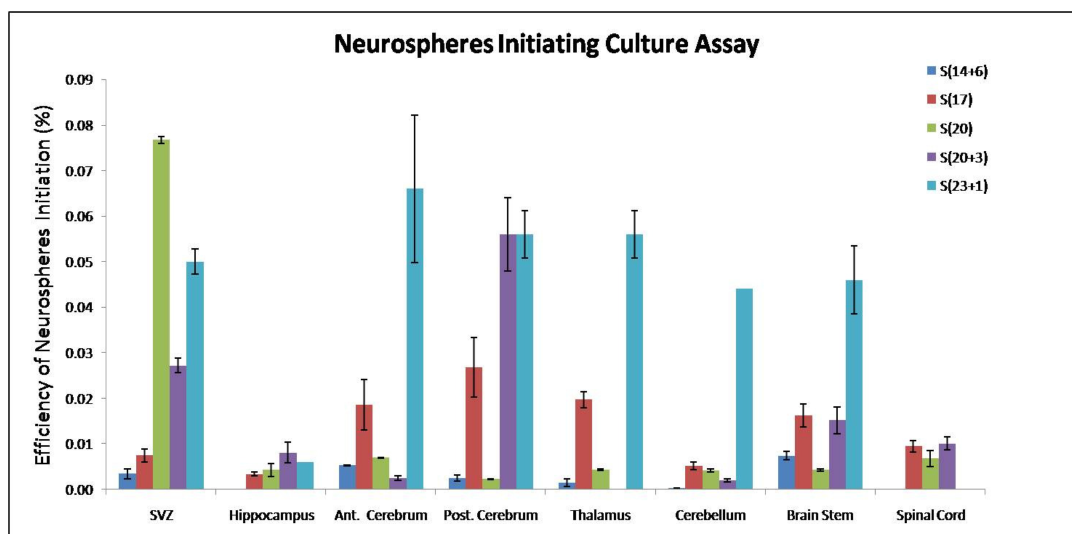
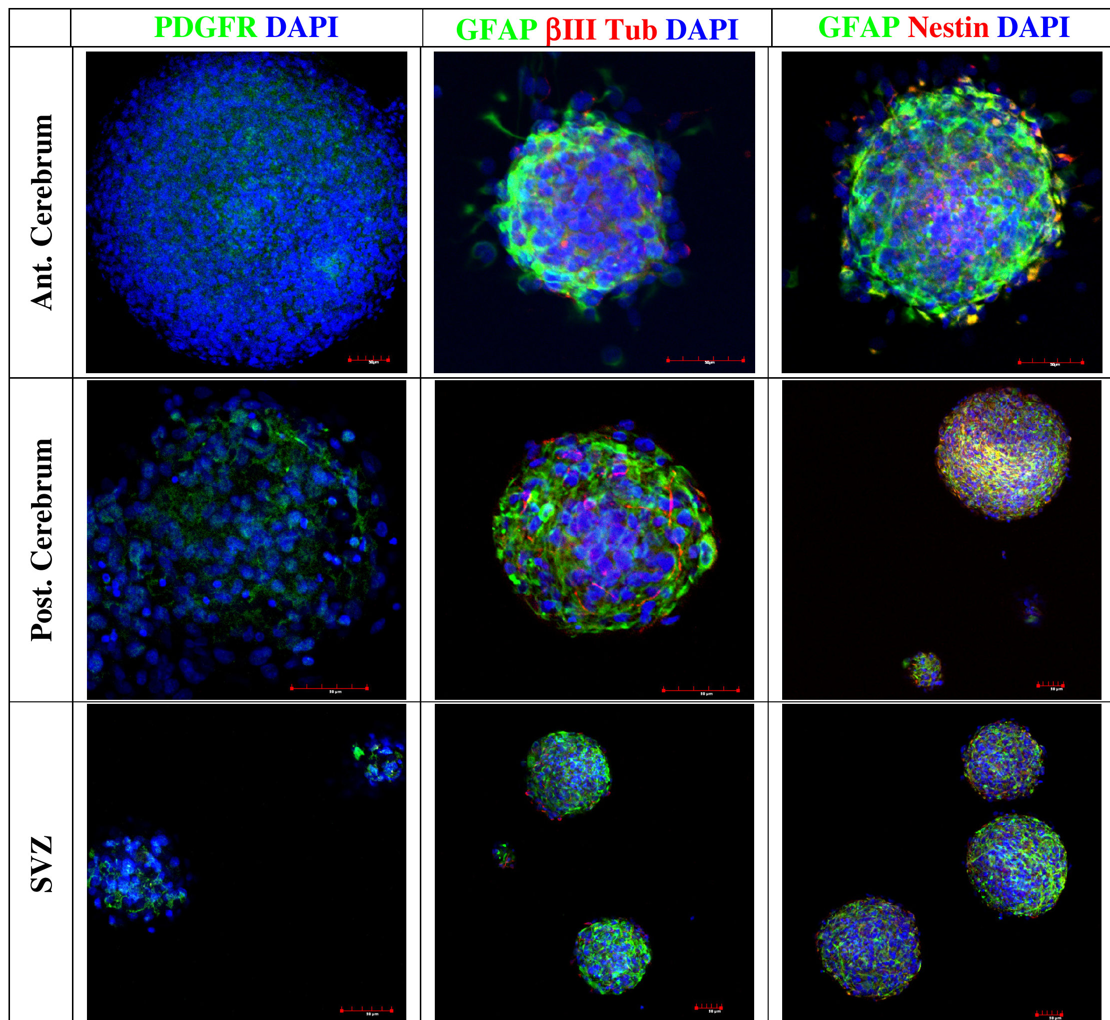


Figure 5-7 Neurospheres-initiating efficiency of cells derived from various regions of the fetal brain. Neurospheres initiating efficiency rises with increasing gestations.

To examine cell-specific antigen expression on the neurospheres, we allowed the neurospheres to attach on poly-lysine coated cells for four hours at 37°C before fixing and staining them. We observed across the neurospheres, positive staining for nestin, Β-tubulin III, GFAP as well as PDGFRα, ascertaining the heterogeneity of cell composition in neurospheres (Figure 5-8).



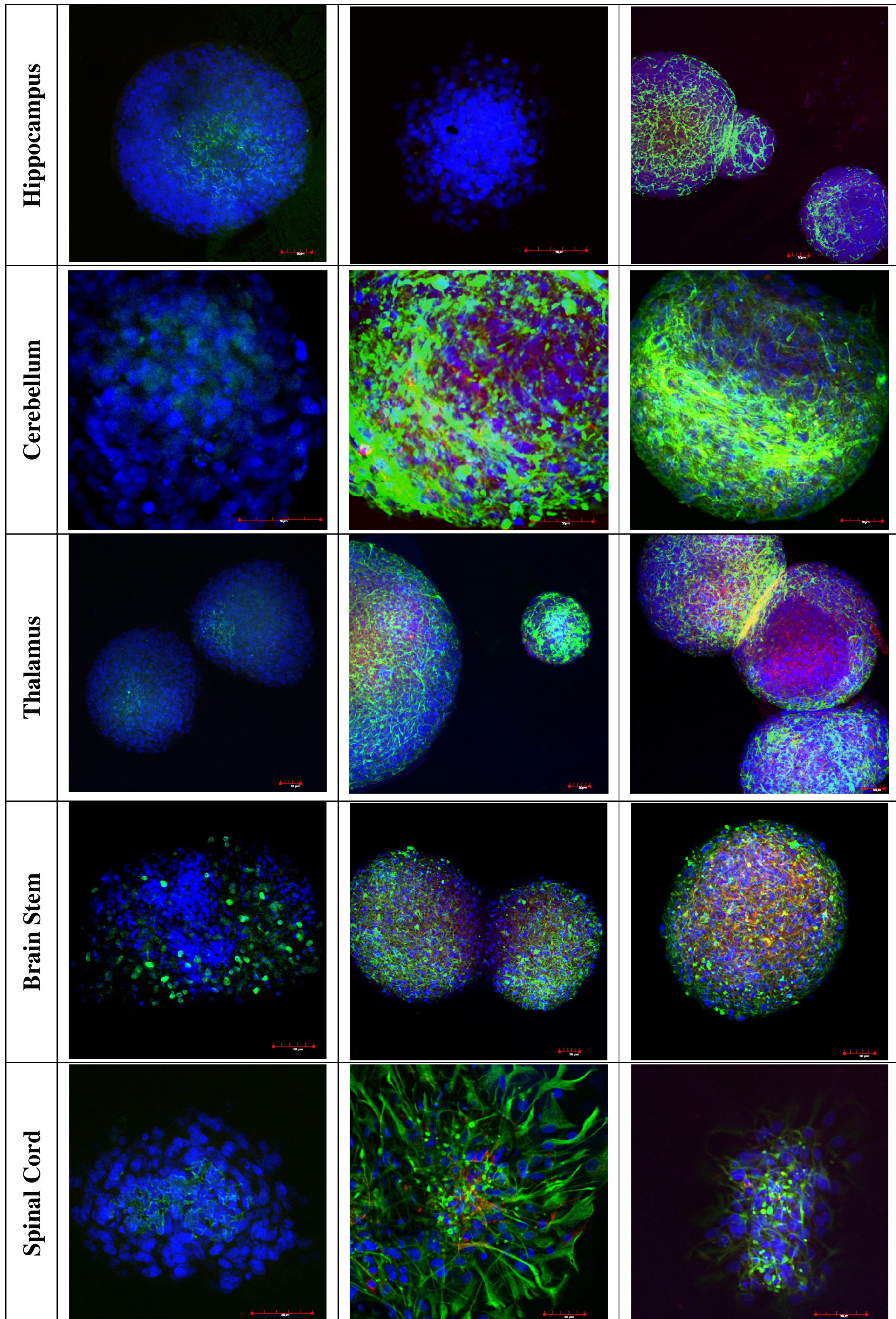
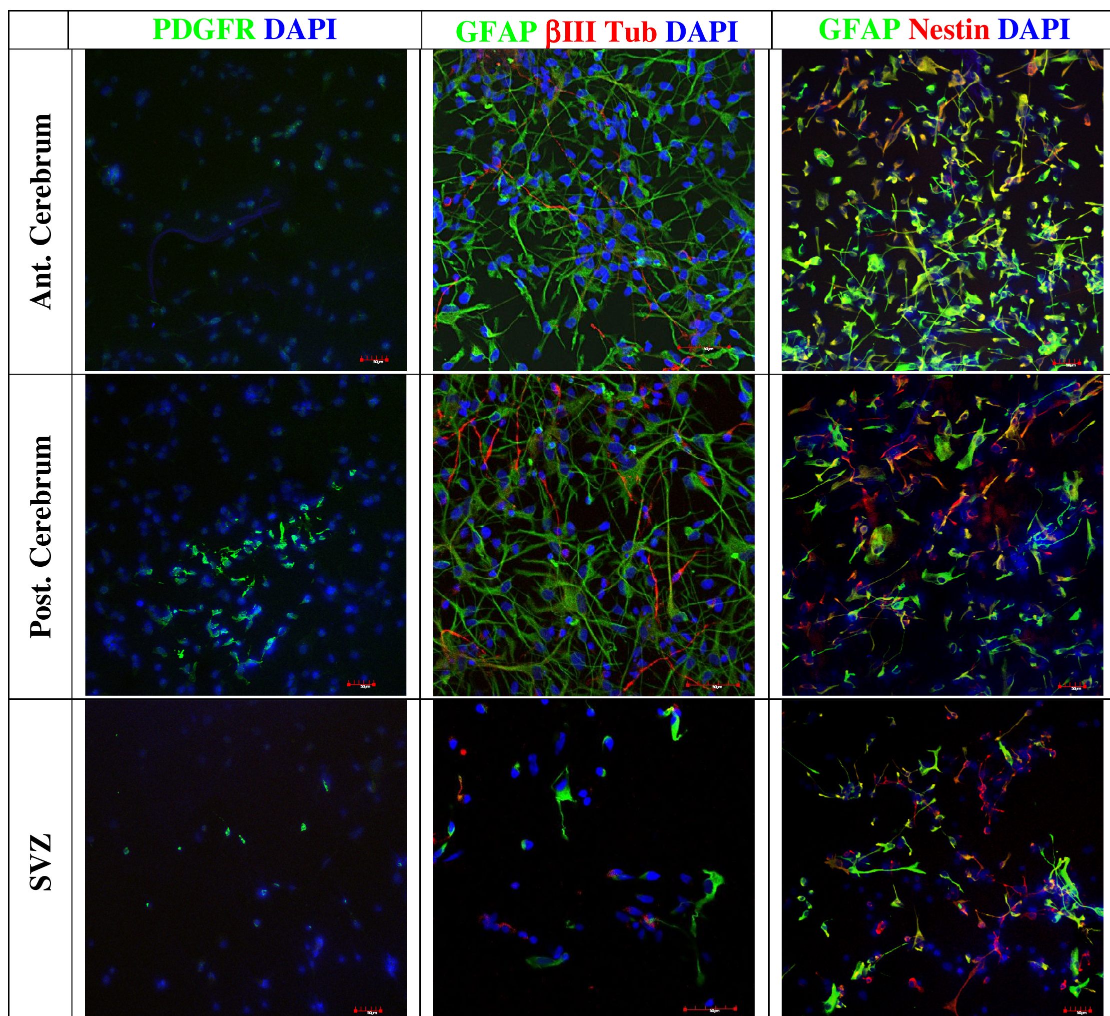


Figure 5-8 Immunocytochemical staining of neurospheres. Cells within the spheres

stained positive for the three known differentiated cells as well as for neural stem cells. Scale: 50µm.

I next differentiated the cells by plating them onto poly-L-ornithine coverslips in medium containing 1% FBS and no mitogens for one week. I observed the spreading of the cells onto the coverslips with short processes branching out and a week later, an entire network of cells intertwined. The cell types of these cells were confirmed by staining with β-Tubulin III, GFAP and PDGFRα (Figure 5-9).



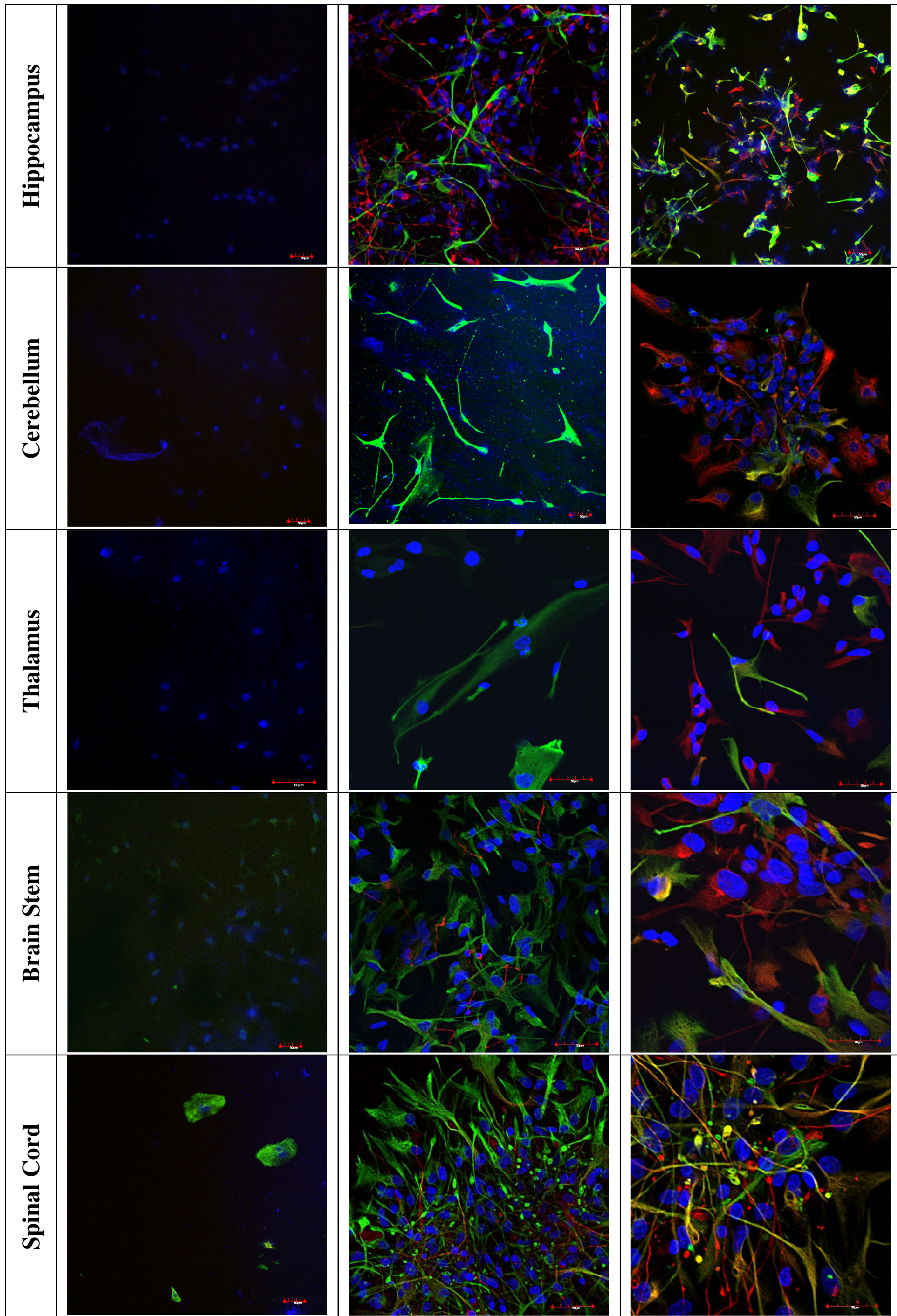


Figure 5-9 Differentiated cells from neurospheres derived from various regions of fetal brain. Cells stained for GFAP, βTubulin III, PDGFRα and nestin. Scale: 50μm.

5.4.1.1.2 Culture of mammospheres from MCF-7

In addition to the use of fetal neural tissues for the generation and characterisation of neural stem cells, I also attempted to isolate mammosphere cultures using a breast carcinoma cell line, MCF-7. SP and non-SP cells from MCF-7 cultures (Figure 5-10a) were singly-sorted into serum-free mammosphere culture medium, where free-floating mammospheres were observed (Figure 5-10b) from the SP fraction of the cell line, but not from the non-SP fraction. These MCF-7 SP derived mammospheres stained positive for CD49f, CD326, CK18, CK14, CK5 and CK19, and negative for CD45 and CD10, an immunophenotype consistent with MaSC cultured as mammospheres (Figure 5-10c).

This verified two important points, first being that cells sorted through flow cytometry were still viable and capable of proliferating and the second point that the mammosphere culture media I used was able to generate mammospheres from the relevant cells.

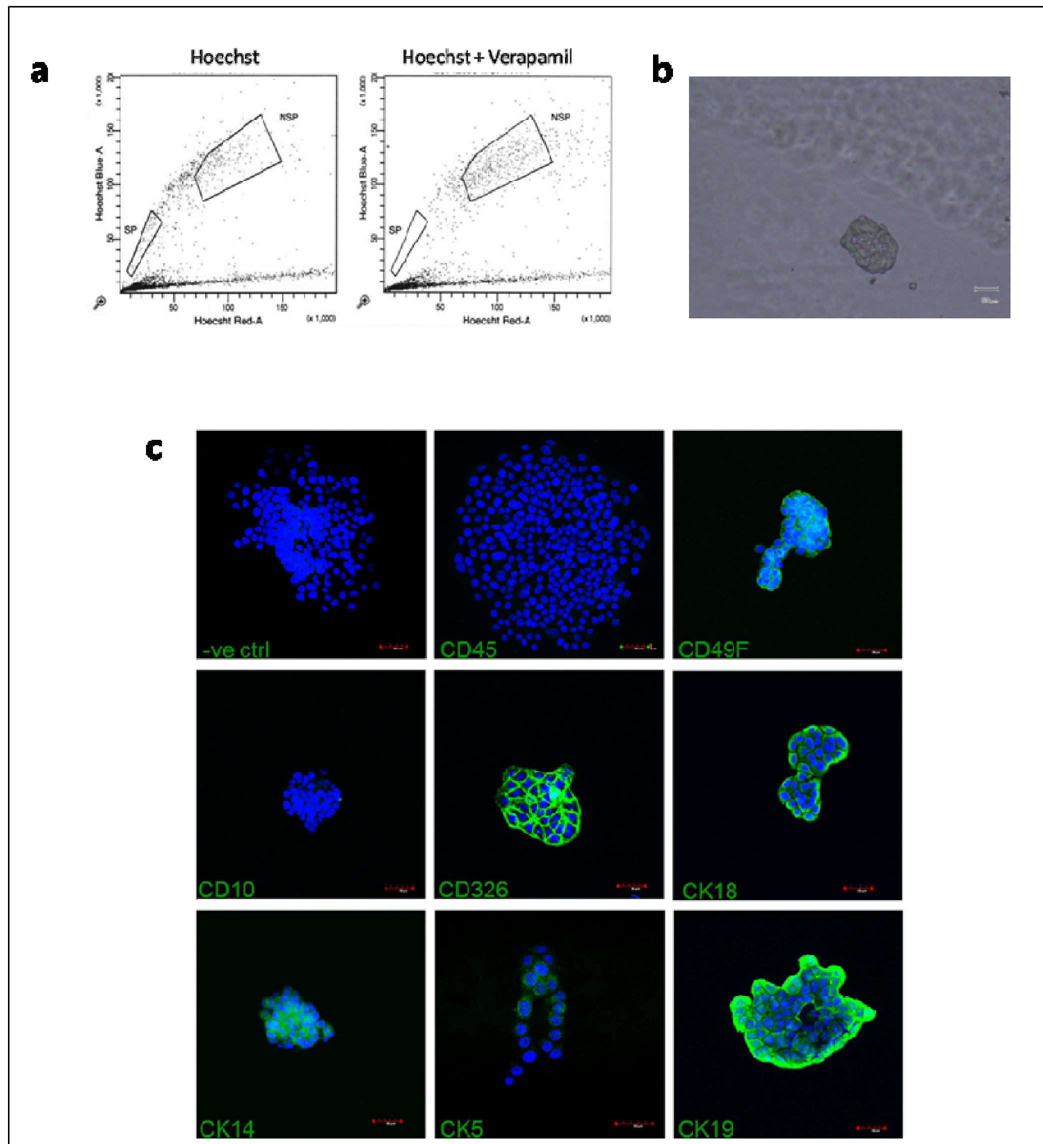


Figure 5-10 Culture of SP of positive control, MCF-7. Flow cytometry profile of MCF-7, a breast cancer cell line from which the SP was sorted out 9(a) grew into mammospheres after ten days (b). Immunocytochemical staining demonstrated that they were CD10 and CD45 negative, and positive for CK5, CK14, CK18 and CK19, and positive for alpha-6 integrin (CD49F) and CD326 (c, green stains with DAPI nuclear counterstaining).

5.4.1.2 Single Cell Cultures

Dontu and team found that SP cells obtained from mammary tissues are able to grow clonally (Dontu et al. 2003a). Singly-sorted SP cells were found to be able to form mammospheres which were not replicated in non-SP cells. As such, I attempted to

replicate this finding in SP cells of HBM at clonal densities. SP and non-SP cells were sorted singly into each well of a 96-well plate and incubated in mammosphere medium. I repeated this for four 96-well plates, using four different milk samples. However, all 768 wells failed to generate any mammospheres over a period of four weeks in culture.

5.4.1.3 Low Density Cell Cultures

Next, I investigated whether the culture of higher densities of SP cells may allow the generation of mammospheres. This approach may allow paracrine factors to assist in the generation of mammospheres, as found in other culture systems for instance, MSC systems which rely on paracrine signalling for the rapid log phase growth (Gregory et al. 2003). Culturing of HBM SP and non-SP cells at a low density of $3x10^3$ cells/ cm^2 on low-adherence 96-well plates however, did not generate any mammospheres over four weeks. Further to this, I investigated the effects of different growth factors and substrata, which may contribute towards the successful expansion of these sorted cells.

5.4.1.4 Optimisation of Expansion of Sorted Cells

5.4.1.4.1 Use of growth factors

Growth factors are proteins that bind to receptors on the cell surface, with the primary result of activating cellular proliferation and/ or differentiation. Epidermal growth factor (EGF) and basic fibroblast growth factor were chosen as they are already being used in mammosphere medium. In additon, EGF receptors have been found on mammary cells (Smith et al. 1984; Smith et al. 1989).

Fetal bovine serum contains a variety of mitogenic growth factors known to aid growth of cells and is routinely used in culture medium.

The niche effect or microenvironment also plays an important role in stem cell biology (Fuchs et al. 2004). To simulate the signalling of a typical niche of these SP cells, conditioned medium gathered from lactocyte culture were added into the mammosphere medium at various concentrations (Table 5-2). However, despite daily inspection of the wells over 2 weeks, no increase in cell numbers to suggest the expansion of the SP cells was observed. Cells did not adhere and blebbing of the cell membrane characteristic of apoptosis was observed (Kerr et al. 1972; Wyllie et al. 1980).

I repeated the sets of single cell and low density culture experiments, with modifications to the mammosphere medium, altering serum and other growth factors as indicated in Table 5-2, with the aim of successful optimisation of culture medium for growth of SP cells in WCP in HBM. However, none of these approaches were successful in the isolation of mammospheres. Reductions of AlamarBlue® was quantitated for each variable in the culture media as shown in Table 5-2 and compared to the mitomycin-treated cells, where no difference was observed, suggesting the lack of cellular proliferation.

Substances added into mammosphere medium					
Fetal Bovine Serum (%)	1	3	5	10	20
Epidermal Growth Factor (ng/ ml)	20	40	60	80	100
Basic Fibroblastic Growth Factor (ng/ ml)	20	40	60	80	100
Conditioned Medium (%)	1	5	20	50	80

Table 5-2: **Substances for optimisation of culture medium.** The four substances, believe to aid cell growth were added in increasing amount as indicated above to optimise the growth of SP cells from HBM.

5.4.1.4.2 Use of substrata

Within the mammary tissue, the various cells lie in an extremely complex ECM. This ECM contains collagens, fibronectin, laminin, glycosaminoglycans and others to interact with the cells, exchange signals and information, allowing the cells to respond accordingly, for instance repair of damaged cells or regeneration of epithelial cells when desired. I used various substrata (matrigel, gelatin, collagen and fibronectin) to line the bottom of the wells on which SP cells were grown. However, despite culturing them for up to 30 days, no proliferation under daily visual inspection was observed (Figure 5-11).

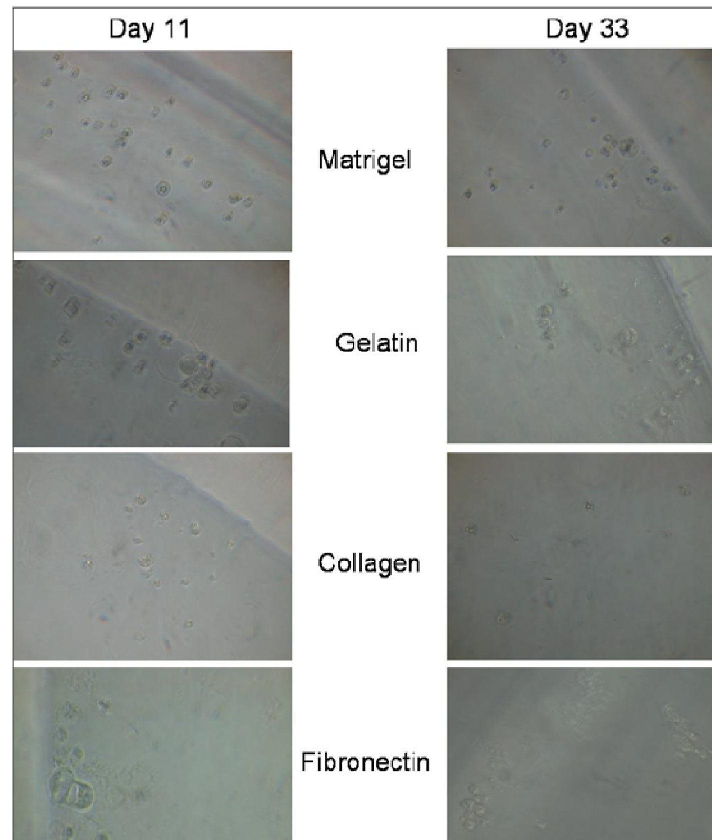


Figure 5-11 Optimisation of culture medium using various substrata. Growth of cells in the various substrata-lined wells at day 11 (left column) and day 33 (right column).

5.4.1.5 Mid Density Cell Cultures

Mid density cell cultures were established by seeding the cells at a higher density of $9.3x10^3$cells/ cm^2 to allow the cells to interact with each other. This is to allow the cells to aid growth synergistically, through exchange of signals. Under visual inspection every two to three days over a period of four weeks, no increase in cell numbers was observed.

5.5 Methylcellulose Culture of Selected Population

5.5.1 Positive Controls for Methylcellulose Cultures

As an appropriate control, I sorted umbilical cord blood for SP cells, and plated them as SP and non-SP cells (Figure 5-12a) in methylcellulose. After two weeks, I observed the development of multi-lineage colony forming units (CFU) from the SP and not the non-SP fraction of umbilical cord blood (Figure 5-12b). This verified two important points; first being that cells sorted from the FACSAria are still viable and capable of proliferating and differentiating, a conclusion synonymous with Section 5.4.1.1.2 and the second point that the methylcellulose culture system I am employing is functional.

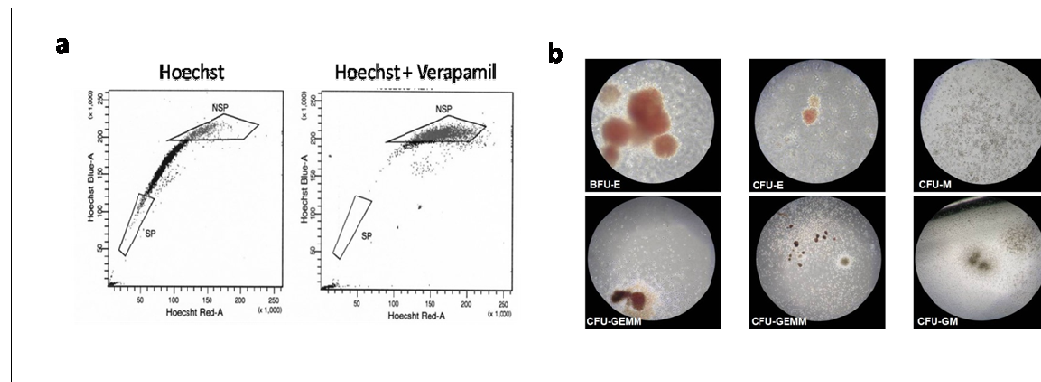


Figure 5-12 Culture of SP from umbilical cord blood cells. Flow cytometry profile of umbilical cord blood with the SP sorted out for culture in methylcellulose (a), confirming the presence of burst forming unit-erythrocyte (BFU-E), colony forming unit-erythrocyte (CFU-E), colony forming unit-macrophage (CFU-M), colony forming unit-granulocyte,erythrocyte, macrophage, megakaryocyte (CFU-GEMM) as well as colony forming unit-granulocyte, macrophage (CFU-GM) in the SP of cord blood (b). Magnification of 4X.

5.5.2 In Vitro Expansion

As the SP fraction in bone marrow is enriched for HSCs (Goodell et al. 1996), and because of the abundance of haemopoietic cells found in HBM, I investigated HBM for the presence of haemopoietic stem cell types, by subjecting the sorted cells into the above methylcellulose culture system. SP and non-SP fractions of HBM were cultured in methylcellulose to induce the formation of haemopoietic colonies. However, I did not find any expansion into haemopoietic colonies (n=5) from either SP or non-SP cellular fraction of HBM.

5.6 Discussion

5.6.1 Summary of Results

In this chapter, I investigated the presence of SP cells within HBM and used it as a putative marker of primitive cell types. SP cells from HBM are enriched for primitive nestin-positive cells and depleted of more mature epithelial cell types which were CK18-positive. The presence of SP coupled with nestin-positivity and CK18-negativity suggest their stem/ progenitor cells' identity. Using flow cytometry, I attempt to look for differences between the SP and non-SP fraction for mammary stem cell, mammary epithelial and even non-epithelial markers. I found minimal differences between the two populations of cells except EPCAM and CD31 that are statistically significant (p-value of 0.02 and 0.04 respectively). I next investigated the cells' potential to self-renew and differentiate down lineages. However, despite attempting to grow mammospheres at single cell, low-density and mid-density, no mammospheres were observed. Neither the addition of various growth factors at varying concentrations nor the use of various

substrata successfully yielded the culture of mammospheres. Controls for spheroid stem cell culture using fetal neural stem cells and breast carcinoma cell line, MCF-7 yielded positive results validating the techniques utilised in pursuing this objective. The attempt to delineate the identity of SP cells in HBM using haemopoietic assay confirmed their non-haemopoietic origin.

5.6.2 Critical Assessment

5.6.2.1 Isolation of Cells

I have reported for the first time, the presence of SP cells in HBM. This Hoechst efflux phenomenon has proven to be a valuable strategy for isolating stem/ progenitor cells from various tissues which do not yet have any established cell surface markers. Cells with an SP phenotype have been described in many tissues including skeletal muscle, lung, liver, heart, testis, kidney, skin, brain and also the mammary gland (Challen and Little 2006). With the isolation of stem/ progenitor cells by Hoechst dye exclusion being carried out successfully on so many adult stem cell types including mammary, I set out to characterise these cells for stem/ progenitor markers and then to confirm their identity through tests for self-renewal and mutli-lineage differentiation.

The comparison of the markers expressed on SP and non-SP cells were however, not very different. As I screened through the various known mammary stem cell markers like CD24, CD29, CD44 and CK5 through flow cytometry, none of them turned out markedly different between the SP and non-SP cells. However, the significant differential expression of nestin and CK18 does point towards the primitive nature of these SP cells.

5.6.2.2 Culture of SP Cells

In order to familiarise myself to the culture system commonly employed for growth of MaSC, I started out the culture systems with two positive controls. After having successfully cultured spheroid cell clusters from primary neural stem cells as well as from a epithelial carcinoma cell line, MCF-7, I went on to attempt single cell cultures of the sorted cells into mammospheres, with an aim of proving clonogenicity and self-renewal. The use of single cell cultures of SP cells from tissues of reduction mammoplasties has been reported (Clarke et al., 2005; Dontu et al., 2003a). This together with the known anatomy of the mammary tissues and my positive controls suggested the feasibility of my experimental plan.

However, I did not manage to obtain the anticipated results regardless of adjustments in terms of growth factors, substrata and even cell densities of the cultures, although I confirmed their non-haemopoietic nature through subjecting the SP cells to a semi-solid growth assay. The proliferative and clonogenic ability of the SP cells from mammary tissues, in contrast with the non-SP cells, has been verified by several groups (Dontu et al. 2003a; Clayton et al. 2004; Clarke et al. 2005) and hence, it is likely that the culture system is sub-optimal in this case.

In the final results chapter of my thesis, I explored another stem cell marker which has been used to isolate different primary and carcinoma cell lines.

6 Isolation of Stem/ Progenitor Cells in Expressed Human Breast Milk by CD133

6.1 Introduction

While there are several markers that are associated with stemness, many were not suitable for use to isolate cells-of-interest as they are intracellular. In terms of cell surface markers, CD34 is one of the earlier and more important markers for stem cells, particularly of the haemopoietic lineage (Civin et al. 1990). More recently, CD133, an antigen 120kDa in size with five transmembrane domain glycoprotein, has also been used as both an alternative and/ or in association for haemopoietic stem cells (Yin et al. 1997; Kobari et al. 2001; Handgretinger et al. 2003). From its primary use as a haemopoietic stem cell marker, its expression has since been implicated in the identification of neural and endothelial stem cells, and other primitive cells (Piechaczek 2001). Since it is a primitive cell marker present on haemopoietic and neuro-ectodermal stem/ progenitor cells (Yin et al. 1997; Uchida et al. 2000), I hypothesise that putative HBM epithelial stem cells may express CD133, allowing their prospective isolation. Hence in this chapter, I sought to isolate viable cells-of-interest by means of CD133 which could potentially be primitive stem cells to carry out more characterisation, in particular pertaining to self-renewal and clonogenicity.

6.2 Isolation of Cells-of-interest using CD133-tagged Dynabeads™

6.2.1 Occurrence of CD133+ Cells

Incubation of WCP in HBM with CD133-tagged Dynabeads™ revealed a small but distinct population of cells accounting for less than 1% of the total cell population (Figure 6-1). This was confirmed by direct immunostaining for CD133 (Figure 6-2).

Figure 6-1 Isolation of CD133-positive cells. CD133-tagged Dynabeads^{TM} observed to attach to cells (red arrows) in HBM on phase contrast microscopy.

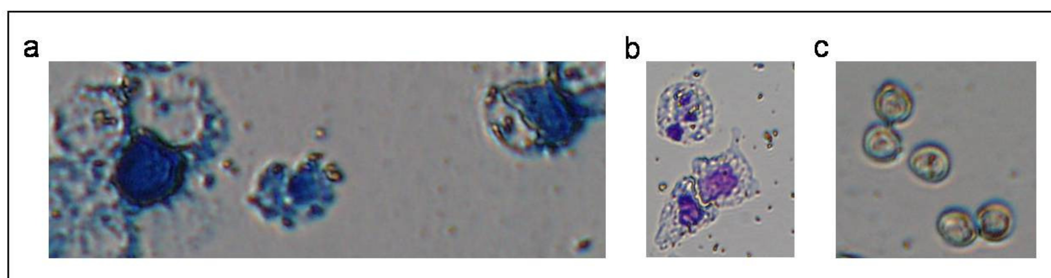


Figure 6-2 Immunocytochemical staining of CD133-positive cells. Cells isolated with CD133-tagged Dynabeads^{TM}, positively-immunostained for CD133 (blue from Vector blue staining) (a) or stained with H & E (b). Erythrocytes were used as external negative control in this instance which were not stained (c).

6.2.2 Growth Kinetics

I tried culturing the cells right after isolation in a 96-well plate but was unsuccessful. Numerous Dynabeads^{TM} remain bound on the positive cells, despite prolonged incubation time with the DNase and the magnetic column. Dynabeads^{TM} isolated CD133+ cells were cultured in epithelial cell culture media for two weeks (n=5) but did not result in any proliferation. Dynabeads^{TM} were seen to be bound to the cells despite culturing the cells beyond a week. Reports have established a detrimental effect of attached Dynabeads^{TM} on cell proliferation and metabolism (Tiwari et al. 2003). Hence,

using Dynabeads™ isolated cells may be helpful for identifying and characterising cell types, but not in experimental set-ups where cells are subjected to culture.

6.2.3 Control Study with CD45-tagged Dynabeads™

I hence decided to attempt CD45 isolation of the immune cells from HBM to ascertain the reliability of this technique for isolation of cells. CD45-tagged Dynabeads™ was not able to deplete the HBM of leukocytes, leaving a large majority of CD45-positive leukocytes within the "negative" fraction, and hardly any cells in the tagged fraction (Figure 6-3).

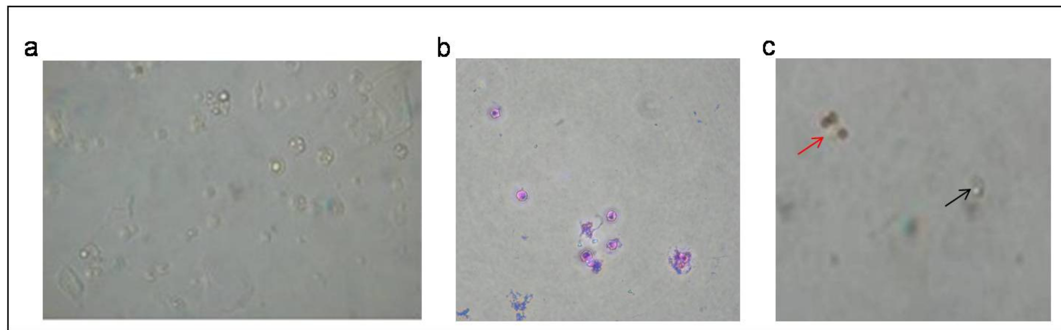


Figure 6-3 CD45-tagged Dynabeads™ isolation of cells from HBM. The negative fraction of the isolation (a-b) was found to still contain a lot of CD45+ lymphocytes (b stained with H&E). The positive fraction (c) hardly had any cells (arrows), some of which were found to be still bound to the Dynabeads™ (red arrow). Magnification of 10X.

6.2.4 Discussion

The isolation of CD45+ cells, which comprises up to 90% of cells in HBM was of extremely low efficiency. MaSC derived from tissue occur at a frequency of 4 in 1000, based on the self-renewal property exhibited through mammosphere formation (Dontu et al. 2003a). It is expected that the frequency of MaSC in HBM would be several log

folds lower than this. Such low proportion of cells-of-interest increases the unreliability of this method for optimal isolation, questioning the purity of cells isolated. Moreover, the continued attachment of CD133 tagged-Dynabeads^{TM} to the putative stem cells over seven days would have impeded the ability of these cells to proliferate or function. A more efficient system using fluorescent-tagged antibody may overcome these problems.

6.3 Isolation of Cells-of-interest using CD133 Antibody Tagged with Fluorescence

6.3.1 Occurrence of CD133+ Cells

Using RPE-tagged CD133 antibody from Miltenyi, I found that 2.0±0.003% (n=23) of the cellular component in HBM are CD133+ (Figure 6-4), which was unaffected by either the maternal age or the duration of breastfeeding (Figure 6-5).

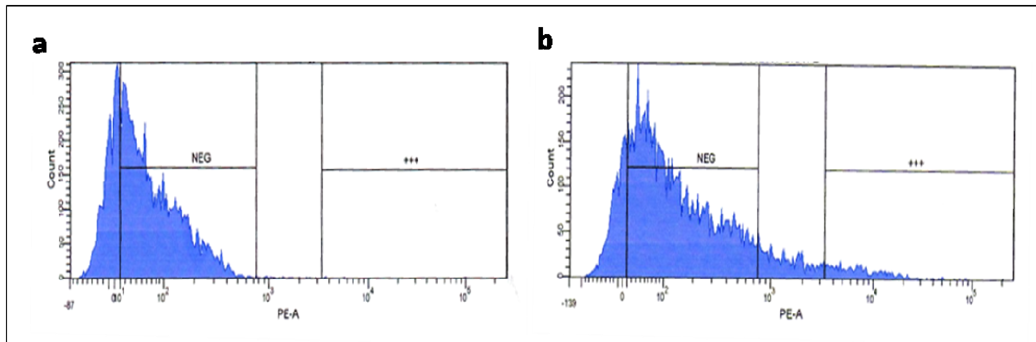


Figure 6-4 CD133 Sorting by FACS. Flow cytometry of CD133 staining on cellular component of HBM (a), as compared to the isotype control (b).

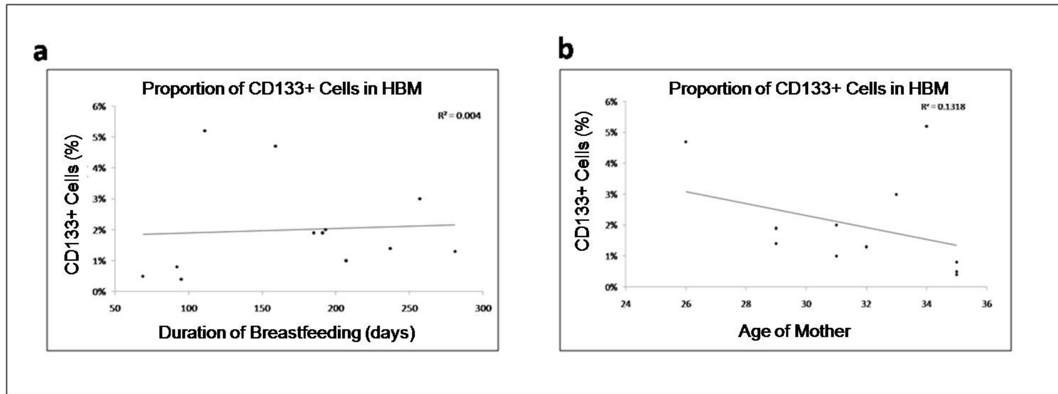


Figure 6-5 Correlation Analysis of CD133+ Cells. There were no relationship between the frequency of CD133 cells and the duration of breastfeeding (a), nor the age of the mother (b).

6.3.2 Characterisation of CD133+ Cells

CD133-positive HBM cells did not demonstrate any significant differences in their expression of EPCAM, CD34, CD45, lineage and CD105 with CD133-negative HBM cells (Table 6-1). This indicated that sorting through the surface marker, CD133, enriched for neither the mammary epithelial cells nor cells of the non-epithelial lineage.

Properties	Antigen	Expression on CD133+	Expression on CD133-	*P*-value
Mammary	EPCAM	1.9 ± 0.98	1.3 ± 0.50	0.63
	CD45	4.2 ± 1.86	8.2 ± 2.10	0.21
Non-epithelial	Lin	0.5 ± 0.19	1.1 ± 0.57	0.36
	CD105	2.8 ± 1.34	11.7 ± 1.84	0.06
	CD34	0 ± 0	0.48 ± 0.38	0.36

Table 6-1: **Antigens expressed on CD133+ and CD133- cells in HBM.**

6.3.3 Mammosphere Culture of CD133+ Cells

Both CD133-positive and CD133-negative HBM cells did not generate any mammospheres in serum-free growth conditions.

6.3.4 Methylcellulose Culture of CD133+ Cells

6.3.4.1 Positive Control

As an appropriate control, I sorted the WCP from umbilical cord blood for Hoechst dye exclusion and then plated the sorted CD133-positive cells into the same methylcellulose system. CD133-positive fraction of umbilical cord blood demonstrated multi-lineage haemopoietic colony forming capacity after two weeks, validating our sorting protocol as well as methylcellulose culture system (Figure 6-6).

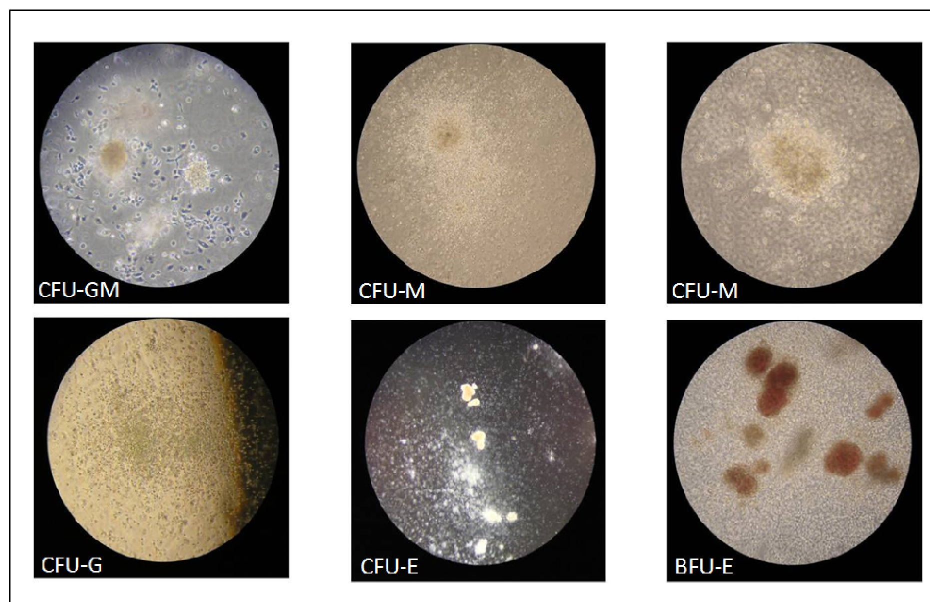


Figure 6-6 Methylcellulose Culture of CD133+ Cells from Cord Blood. CD133+ cells from cord blood formed multi-lineage colonies on CFU assays. Magnification of 4X.

6.3.4.2 In Vitro Expansion

As the CD133 fraction in bone marrow and cord blood is enriched for haemopoietic stem cells (Charrier et al. 2002; Yao et al. 2006; Lu et al. 2007), I investigated CD133-positive cells in HBM for the presence of haemopoietic stem cell types in the same methylcellulose culture system mentioned earlier in Section 5.5. Similar to the SP and non-SP fractions of HBM, I did not find the expansion of any haemopoietic colonies (n=3) in either the CD133-positive or CD133-negative cellular fraction from HBM.

6.4 Discussion

6.4.1 Summary of Results

In this chapter, I found no more than 2% of CD133-positive cells in HBM, a phenomenon never described nor published before. Two methods, namely the use of Dynabeads™ and flow sorting with fluorescent-tagged CD133 antibody were employed with the latter found to pull out a population of CD133-positive cells which was more suitable for culture purposes.

With regards to the characterisation by flow cytometry, there was no significant difference between the CD133-positive and CD133-negative cells (p-value > 0.05). This is perplexing as the difference turned out more insignificant compared to the difference between SP and non-SP cells.

I subjected the CD133-positive and CD133-negative cells to methylcellulose as well as mammosphere culture system and both, similar to the SP cells did not have any colonies or mammospheres forming respectively.

6.4.2 Critical Assessment

6.4.2.1 Isolation of CD133+ Cells

Initially, I made use of CD133-tagged Dynabeads™ to selectively pull out the potential stem/ progenitor cells. Upon problems of high cell loss (positive cells being still present in the negative fraction) and the large Dynabeads™ still attached onto the sorted cells, I believe it would be fruitful to use the same method of CD133 isolation but with a different kit. FACS was then employed for its high reliability and efficiency. Therefore, I planned to carry out isolation of CD133-positive cells using fluorescent-tagged CD133 antibody, characterise them and examine their proliferation behaviour in various culture systems.

6.4.2.2 Characterisation of CD133+ Cells

CD133-positive cells in HBM are completely novel. It has been reported on mammary tumours (Wright et al. 2008) raising the question of link between the cells found in HBM and the tumorigenesis of mammary cells, resulting in malignancies. Hypothetically, these cells in HBM could serve as a potential source for research groups to study the transformation of mammary cells and the changes involved, which in the long run could bring about the better understanding and possibly even novel therapies for mammary cancers.

The simple characterisation of these cells for EPCAM and other non-epithelial cell markers however did not provide further clues to their identity as from the flow cytometry results, there appear no difference between the CD133-positive and CD133-negative cells. I therefore went ahead with the mammosphere and methylcellulose

culture so that I can conclude if there are mammary and/ or haemopoietic stem/ progenitor cells in either population. With the negative culture results and a positive control that validate my sorting and culture protocol, I can certainly conclude the absence of mammary and haemopoietic stem/ progenitor cells in HBM within my culture parameters.

7 General Discussion

7.1 Introduction

The search and use of stem cells in the human has been under the spotlight particularly in recent years. Sources for haemopoietic stem/ progenitor cells are plentiful and many of which are already in use for clinical transplantation, proving their functionality and widespread potential. However, demand still far exceeds supply and hence, we still have many research groups around the world working on the in vitro expansion of these cells and coming up with novel sources like making of artificial blood to circumvent the shortage problem. HBM has previously been shown to contain a large amount of haemopoietic cell types, but it has never been explored as a source of HSC.

MaSC are also under stringent scrutiny in recent years, due in no small part to the fact that breast cancer is the most common cancer among females worldwide and that MaSC have been implicated in the progress of this prevalent disease (Dontu et al. 2003b; Dontu et al. 2005; Ponti et al. 2005). While MaSC have been obtained from mammary tissues, this involves an invasive procedure which limits its accessibility. Based on studies which have proved that stem cells are discharged into luminal cavities, in the case of mesenchymal progenitor cells in menstrual blood and epithelial, urothelial and smooth muscle lineage stem/ progenitor cells in urine (Hida et al. 2008; Musina et al. 2008; Zhang et al. 2008), I proposed the question of MaSC being present in HBM, which is the main aim of my study. As a side question, I also decided to investigate HSC, given that the main cellular component of HBM is immune cells, responsible for much of the beneficial effects conferred to the baby.

In my study, I aimed to isolate MaSC and HSC from HBM, believing that with successful derivation of these cells from HBM, it will aid progress of research in these two fields and provide a novel and non-invasive source of mammary and/ or haemopoietic stem/ progenitor cells. In addition, this would allow a more detailed and complete understanding of the various components of HBM, possibly even unravelling more unknowns about HBM.

7.2 Hypotheses

The aim of this project was to determine whether there are any adult stem/ progenitor cells present in HBM and that if they are present, whether they are able to differentiate down various epithelial and/ or haemopoietic lineages, demonstrating multipotency. This would potentially have implications for breast tissue engineering, clinical application for haemopoietic cell use and for elucidation of the mechanisms between MaSC and progression to malignancy as well as the mechanism of lactogenesis. In Section 1.5.1, I have hypothesised that adult stem/ progenitor cells are present in HBM and that they are able to differentiate down various lineages and perhaps even transdifferentiate. Here, I set off answering my questions by (i) searching for markers of various lineages, (ii) culturing the adherent cells to get a purer population of cells to work with in search of my cells-of-interest and lastly, (iii) the use of specific markers in an attempt to isolate the cells of my interest.

7.3 Findings

7.3.1 Cellular Component of Expressed Human Breast Milk

I have discovered the presence of cell surface markers that have never been reported in the cellular component of HBM. The presence of these markers suggests the presence of one or more novel cell types in HBM. Specifically, there was positive staining of stem/ progenitor markers of various lineages like ABCG2, CD24, β-1 and α-6 integrins, all of which have been implicated in the identification of MaSC (Shackleton et al. 2006; Stingl et al. 2006a, b).

The presence of a large number of IFs were investigated with flow cytometry and the positive expression of CK5 and the neural IFs once again suggests the presence of a yet still uncharacterised cell population in HBM.

7.3.2 In Vitro Expansion of Adherent Cells in Selective Medium

I have managed to circumvent the problem of staining WCP in HBM by expanding the adherent cells for up to 30 days. These cells were identified as epithelial cells by epithelial specific immunocytochemistry. From their growth kinetics, it is apparent that they demonstrate great variability in doubling time and rate of proliferation.

In chapter 3, I have observed the presence of CK5 RNA expression in WCP of HBM. However, in chapter 4, when I directly compared the expression level of CK5 and CK14 by means of RT-PCR, it was relatively much lower, so much so that they are beneath the threshold level of positivity. In the case of cultured cells, I observed nestin and CK5 positivity and a co-localisation of the 2 IFs in 11.4±4.2% of cells. The pattern

of staining seems to suggest the presence of a hierarchy of cells within the lobulo-alveoli structure which are being sloughed off into HBM with the nestin-positive cells being the multipotent progenitor cells that differentiate to express CK5 as the mammary stem/ progenitor cells which further differentiate into the known luminal epithelial, myoepithelial and ductal cells.

7.3.3 Attempts to Isolate the Stem/ Progenitor Cells

7.3.3.1 Hoechst Dye Exclusion

The method of Hoechst dye exclusion is a method employed by many stem cell groups to isolate a side-population of cells which possess stem cell properties like self-renewal, clonogenicity and the ability to differentiate into mature cell types. In HBM, I found a proportion of approximately 2.1% of cells forming the side-population. The proportion of side-population appears to be unaffected by the duration of breastfeeding. SP was highly nestin-positive and not CK18, suggestive of their primitive identity. Further to this, flow cytometry exploring the differences between SP and non-SP for a series of other markers indicative of MaSC, mammary epithelial cells as well as non-epithelial cells concluded minimal difference with exception of EPCAM and CD31.

With appropriate controls in place, it was found that neither SP nor non-SP cellular fraction contain mammary or haemopoietic stem/ progenitor cells that could be cultured. It might be due to the insufficiencies of the medium, although I have proven that addition of EGF, bFGF, FBS or conditioned medium does not improve the culture conditions for mammary or haemopoietic stem/ progenitor cells in this setting. There

remains a need to exhaustively try out different medium and perhaps an in vivo transplantation strategy.

7.3.3.2 CD133

I attempted the isolation of CD133-positive cells through two different means before finalising the use of fluorescence-tagged antibodies, which was preferred due to the pure selection, higher efficiency of sorting and suitability for culture.

Comparable to SP cells, I found a proportion of 2.0% of cells that are CD133-positive and this proportion is unaffected by both the maternal age and the duration of breastfeeding. While CD133 and SP picked up the same proportion of cells, it is noteworthy to mention that they are unlikely to be the same cells as I have done a comparison of the SP and non-SP for expression of CD133, which I found to be 6.1±1.9 and 3.9±2.0% (Table 5-1, page 116). Characterisation of CD133-positive and CD133-negative cells to discover any differences in the other cell surface markers they possess, namely EPCAM, CD45, Lin, CD105 and CD34, did not show any significant difference.

Neither CD133-positive and CD133-negative cells generated mammospheres or colonies in the mammospheres and methylcellulose cultures, once again concluding that there are neither mammary nor haemopoietic stem/ progenitor cells that can be cultured from HBM.

7.4 Limitations

This project proved to be more challenging than initially envisioned. Along the way of answering the hypotheses, several other factors limiting further research are detailed as follows.

Where the recruitment of participants was concerned, I had intended to recruit from just one institution. But in the first year, I faced great challenges in liasing with potential volunteers in the first few days postpartum when their milk supply has yet to be established and I had difficulties getting them enrolled. In addition, there were issues with the volume of HBM that they are comfortable with donating. I first started out with a mere 10 to 15mL per donor which yielded a hardly visible cell pellet to work with. In the following year, I managed to get increased donations from lactating women through the various breastfeeding support groups, in particular the Singapore Motherhood Forum. Through these support groups, I managed to involve a larger number of women who individually are able to contribute larger volumes of HBM.

In attempting to characterise the cellular component of HBM, the high background staining of uncultured cells proved challenging. Despite optimising the staining protocol with increased incubation with blocking agent, by altering the components of the blocking agent and adjusting the incubation times with antibodies and even antibodies against same antigens purchased from different companies, a high background staining still existed. I moved to show the presence of the various lineage markers through RT-PCR and flow cytometry which allows the right gating of single cells as well as rapid analysis of at least 20,000 single cells within a short period of time.

In the search for an appropriate means to isolate my cells-of-interest, a major limitation was the lack of suitable markers. Even if the cells-of-interest were to be MaSC, the markers to identify them were not definitive. The most definitive work on MaSC was in my opinion that of Shackleton and team where they managed to generate a functional mammary gland from a single mammary stem cell based on the marker of Lin-CD29hiCD24+ (Shackleton et al. 2006). However, the same markers are also used for identifying white adipocyte progenitors in vivo (Rodeheffer et al. 2008). CK5 has also been named as a mammary stem cell marker (Bocker et al. 2002; Dontu et al. 2003a) but it has also been implicated in the identification of epitheloid mesothelioma (Clover et al. 1997) and epidermis (Mischke and Wild 1987).

Finally, with the negative results I got in the culture components after sorting, the controls in place had to be extremely exhaustive ranging from fetal tissue to umbilical cord blood to cell lines which each had its own difficulty in terms of availability. Nonetheless, it was a great chance to work with a wide variety of tissues, an opportunity not many researchers get, since such tissues are scarce and not usually available.

Although not entirely exhaustive, but certainly within the confines and limitations of my graduate study, an in vivo experimental system may be a better approach in identifying putative stem cells, if they are present, as evidently the in vitro conditions has not been well defined as yet.

7.5 Implications of This Research

The significance of a positive result for this work is profound as mentioned in Section 1.5.2. Besides providing novel cell sources for breast tissue engineering and potential banking of stem cells from all lactating females, a positive result would enable deeper understanding of the synergistic relationship breastfeeding has on the mother and infant. MaSC from HBM could aid to elucidate mechanisms that brings about mutagenesis and oncogenesis, which breastfeeding has been shown to exert a protective effect (Byers et al. 1985; Katsouyanni et al. 1986; Newcomb et al. 1994; Brinton et al. 1995).

In addition, it is well studied that fetal cells have been found in maternal blood and organs up to 30 years after birth (O'Donoghue et al. 2004; Tan et al. 2005; O'Donoghue et al. 2008) but less is known of the reverse whereby maternal cells cross the placenta and implant in perinatal tissues. If HBM contains stem or pluripotent cells, do these cells implant in the infant and are the transportation of these maternal stem cells to the breastfeeding infant associated with possible benefits (increased population of progenitors that assist with repair of damaged tissues) or deleterious effects to the infant by causing graft versus host reactions, autoimmune processes or stem cell derived neoplasia? Afterall, it has already been shown in various models that leukocytes from milk escape digestion in the infant’s gut and enter the circulation of the infant (Weiler et al. 1983; Slade and Schwartz 1987; Jain et al. 1989; Arvola et al. 2000).

With my results, we can conclude that while there are indeed promising markers, cells isolated with these markers were not capable of proliferating in vitro within my experimental parameters. This can be explained with the fact that my culture conditions, although optimised with a variety of growth factors and substrata remains sub-optimal.

The second explanation would be that stem/ progenitor cells though present in the mammary gland are not passed out into HBM. With respect to the latter scenario, there are certainly specific mechanisms within the human which prevents the sloughing of such important cells of our system. Instead, perhaps only cells that are meant to be replaced or meant for the baby's immune system are sloughed off into HBM. Clearly, mammary and haemopoietic stem/ progenitor cells are not in the list.

With my results, we have answered one important biological question asked not just by us and it will perhaps, conclude the search for these much-sought after cells in HBM, which was intitally viewed by us and others as a potential novel non-invasive source of such cells (McGregor and Rogo 2006; Cregan et al. 2007).

7.6 Directions for Future Research

7.6.1 Isolation of Cells-of-interest

As mentioned earlier, Shackleton and team demonstrated that cells from reduction mammoplasties contained within the Lin-CD29hiCD24low population are able to reconstitute an entire functional mammary gland (Shackleton et al. 2006) and that this is, by far, demonstration at the highest level of what would constitute a true stem cell, at least in the murine model. CD29 and CD24 have been reported to be present on MaSC in both mouse and human (Shackleton et al. 2006; Stingl et al. 2006b). Therefore, it might be worthwhile to triple select for Lin-CD29hiCD24low cells and attempt a mammosphere culture. Removal of haemopoietic lineages from WCP from HBM (Rossette-separation, StemCells) by magnetic sorting can be pursued, following which, flow-cytometric sorting of CD29+ and CD24+ cells by dual-colour sorting can be

carried out. As was done with the cells isolated by the other methods, these cells can be characterised and put into cultures to find out their proliferative ability, clonogenicity and ability to self-renew.

7.6.2 In Vivo Work

To prove with absolute certainty, one should transplant these cells from HBM into fatpad removed nonobese diabetes/severe combined immunodeficiency mice. To do this, one would also be required to optimise the cell numbers to be transplanted and the length of observation.

7.7 Conclusions

During the course of my studies for this thesis, I have established, for the first time, the presence of stem cell markers like nestin, CD133, and the presence of SP cells in expressed HBM. Contrary to my hypothesis, cells expressing these markers, which are often considered characteristic of stem/ progenitor cells, could not be cultured using the standard stem cell culture techniques I have employed. Although not exhaustive, the culture methods I have used were all I could perform within the confines and limitations of my graduate studies. An in vivo experimental system may therefore be necessary to identify the putative stem cells, if indeed they are present, as I have proved that standard in vitro culture methods are not able to grow any stem cells from HBM.

References

(2002). "Morality, prejudice and cloning." Nature **415**(6870): 349.

Adams, R. B., S. M. Planchon and J. K. Roche (1993). "IFN-gamma modulation of epithelial barrier function. Time course, reversibility, and site of cytokine binding." J Immunol **150**(6): 2356-63.

Al-Hajj, M. and M. F. Clarke (2004). "Self-renewal and solid tumor stem cells." Oncogene **23**(43): 7274-82.

Al-Hajj, M., M. S. Wicha, A. Benito-Hernandez, S. J. Morrison and M. F. Clarke (2003). "Prospective identification of tumorigenic breast cancer cells." Proc Natl Acad Sci U S A **100**(7): 3983-8.

Altemus, M., P. A. Deuster, E. Galliven, C. S. Carter and P. W. Gold (1995). "Suppression of hypothalmic-pituitary-adrenal axis responses to stress in lactating women." J Clin Endocrinol Metab **80**(10): 2954-9.

Alvi, A. J., H. Clayton, C. Joshi, T. Enver, A. Ashworth, M. M. Vivanco, T. C. Dale and M. J. Smalley (2003). "Functional and molecular characterisation of mammary side population cells." Breast Cancer Res **5**(1): R1-8.

American Academy of Paediatrics:Breastfeeding and the use of human milk. Pediatrics **100:** 1035-9, 1997

Amoh, Y., L. Li, K. Katsuoka, S. Penman and R. M. Hoffman (2005). "Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons." Proc Natl Acad Sci U S A **102**(15): 5530-4.

Anderson, E. and R. B. Clarke (2004). Mammary epithelial stem cells. Adult Stem Cells. K. Turksen. Totowa, Humana Press**:** pp 191-214.

Antoniou, M. (2001). "Embryonic stem cell research. The case against." Nat Med **7**(4): 397-9.

Aperia, A., O. Broberger, P. Herin and R. Zetterstrom (1979). "Salt content in human breast milk during the three first weeks after delivery." Acta Paediatr Scand **68**(3): 441-2.

Archer, D. L. and H. M. Johnson (1978). "Blockade of mitogen induction of the interferon lymphokine by a phenolic food additive metabolite." Proc Soc Exp Biol Med **157**(4): 684-7.

Arthur, P. G., J. C. Kent, J. M. Potter and P. E. Hartmann (1991). "Lactose in blood in nonpregnant, pregnant, and lactating women." J Pediatr Gastroenterol Nutr **13**(3): 254-9.

Arthur, P. G., M. Smith and P. E. Hartmann (1989). "Milk lactose, citrate, and glucose as markers of lactogenesis in normal and diabetic women." J Pediatr Gastroenterol Nutr **9**(4): 488-96.

Arvola, M., E. Gustafsson, L. Svensson, L. Jansson, R. Holmdahl, B. Heyman, M. Okabe and R. Mattsson (2000). "Immunoglobulin-secreting cells of maternal origin can be detected in B cell-deficient mice." Biol Reprod **63**(6): 1817-24.

Atkinson, S., B. Alston-Mills, B. Lonnerdal and M. C. Neville (1995). Major minerals and ionic constituents of human and bovine milk. Handbook of Milk Composition. R. G. Jensen. New York, Academic Press: pp 593-622.

Atwood, C. S. and P. E. Hartmann (1995). "Assessment of mammary gland metabolism in the sow. III. Cellular metabolites in the mammary secretion and plasma following weaning." J Dairy Res **62**(2): 221-36.

Azuma, N., K. Yamauchi and T. Mitsuoka (1984). "Bifidus Growth-promoting Activity of a Glycomacropeptide Derived from Human K-Casein." Agricultural and Biological Chemistry **48**(8): 2159-2162.

Bae, S. N., G. Arand, H. Azzam, P. Pavasant, J. Torri, T. L. Frandsen and E. W. Thompson (1993). "Molecular and cellular analysis of basement membrane invasion by human breast cancer cells in Matrigel-based in vitro assays." Breast Cancer Res Treat **24**(3): 241-55.

Baker, D., H. Taylor and J. Henderson (1998). "Inequality in infant morbidity: causes and consequences in England in the 1990s. ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood." J Epidemiol Community Health **52**(7): 451-8.

Bates, C. J. and A. Prentice (1994). "Breast milk as a source of vitamins, essential minerals and trace elements." Pharmacol Ther **62**(1-2): 193-220.

Baum, C. M., I. L. Weissman, A. S. Tsukamoto, A. M. Buckle and B. Peault (1992). "Isolation of a candidate human hematopoietic stem-cell population." Proc Natl Acad Sci U S A **89**(7): 2804-8.

Bennett, D. C., L. A. Peachey, H. Durbin and P. S. Rudland (1978). "A possible mammary stem cell line." Cell **15**(1): 283-98.

Bertoncello, I., G. S. Hodgson and T. R. Bradley (1985). "Multiparameter analysis of transplantable hemopoietic stem cells: I. The separation and enrichment of stem cells homing to marrow and spleen on the basis of rhodamine-123 fluorescence." Exp Hematol **13**(10): 999-1006.

Binkley, F., M. L. Wiesemann, D. P. Groth and R. W. Powell (1975). "Gamma-glutamyl transferase: a secretory enzyme." FEBS Lett **51**(1): 168-70.

Bitman, J., L. Wood, M. Hamosh, P. Hamosh and N. R. Mehta (1983). "Comparison of the lipid composition of breast milk from mothers of term and preterm infants." Am J Clin Nutr **38**(2): 300-12.

Bittner, R. E., C. Schofer, K. Weipoltshammer, S. Ivanova, B. Streubel, E. Hauser, M. Freilinger, H. Hoger, A. Elbe-Burger and F. Wachtler (1999). "Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice." Anat Embryol (Berl) **199**(5): 391-6.

Bjerknes, M. and H. Cheng (2006). "Intestinal epithelial stem cells and progenitors." Methods Enzymol **419**: 337-83.

Bocker, W., R. Moll, C. Poremba, R. Holland, P. J. Van Diest, P. Dervan, H. Burger, D. Wai, R. Ina Diallo, B. Brandt, H. Herbst, A. Schmidt, M. M. Lerch and I. B. Buchwallow (2002). "Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept." Lab Invest **82**(6): 737-46.

Boecker, W. and H. Buerger (2003). "Evidence of progenitor cells of glandular and myoepithelial cell lineages in the human adult female breast epithelium: a new progenitor (adult stem) cell concept." Cell Prolif **36 Suppl 1**: 73-84.

Bonnet, D. (2002). "Haematopoietic stem cells." J Pathol **197**(4): 430-40.

Boulanger, C. A., K. U. Wagner and G. H. Smith (2005). "Parity-induced mouse mammary epithelial cells are pluripotent, self-renewing and sensitive to TGF-beta1 expression." Oncogene **24**(4): 552-60.

Bracco, U., J. Hidalgo and H. Bohren (1972). "Lipid composition of the fat globule membrane of human and bovine milk." J Dairy Sci **55**(2): 165-72.

Brinton, L. A., N. A. Potischman, C. A. Swanson, J. B. Schoenberg, R. J. Coates, M. D. Gammon, K. E. Malone, J. L. Stanford and J. R. Daling (1995). "Breastfeeding and breast cancer risk." Cancer Causes Control **6**(3): 199-208.

Brock, J. H. (1980). "Lactoferrin in human milk: its role in iron absorption and protection against enteric infection in the newborn infant." Arch Dis Child **55**(6): 417-21.

Brooker, B. E. (1980). "The epithelial cells and cell fragments in human milk." Cell Tissue Res **210**(2): 321-32.

Broxmeyer, H. E. (2004). Proliferation, self-renewal, and survival characteristics of cord blood hematopoietic stem and progenitor cells. Cord Blood: Biology, Immunology, and Clinical Transplantation. H. E. Broxmeyer. Bestheda, American Association of Blood Banks Press**:** pp 1-21.

Broxmeyer, H. E. (2005). "Biology of cord blood cells and future prospects for enhanced clinical benefit." Cytotherapy **7**(3): 209-18.

Broxmeyer, H. E., G. W. Douglas, G. Hangoc, S. Cooper, J. Bard, D. English, M. Arny, L. Thomas and E. A. Boyse (1989). "Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells." Proc Natl Acad Sci U S A **86**(10): 3828-32.

Broxmeyer, H. E., G. Hangoc, S. Cooper, R. C. Ribeiro, V. Graves, M. Yoder, J. Wagner, S. Vadhan-Raj, L. Benninger, P. Rubinstein and et al. (1992). "Growth characteristics and expansion of human umbilical cord blood and estimation of its potential for transplantation in adults." Proc Natl Acad Sci U S A **89**(9): 4109-13.

Broxmeyer, H. E., E. Srour, C. Orschell, D. A. Ingram, S. Cooper, P. A. Plett, L. E. Mead and M. C. Yoder (2006). Cord blood stem and progenitor cells. Methods in Enzymology. I. Klimanskaya and R. Lanza. San Diego, Academic Press. **419:** pp 439-473.

Buehring, G. C. (1972). "Culture of human mammary epithelial cells: keeping abreast with a new method." J Natl Cancer Inst **49**(5): 1433-4.

Bueno, J., M. Torres, A. Almendros, R. Carmona, M. C. Nunez, A. Rios and A. Gil (1994). "Effect of dietary nucleotides on small intestinal repair after diarrhoea. Histological and ultrastructural changes." Gut **35**(7): 926-33.

Buescher, E. S. and I. Malinowska (1996). "Soluble receptors and cytokine antagonists in human milk." Pediatr Res **40**(6): 839-44.

Buescher, E. S. and L. K. Pickering (1986). Polymorphonuclear leukocytes in human colostrum and milk. Human Milk in Infant Nutrition and Health. R. R. Howell, F. H. Morriss and L. K. Pickering. Springfield, Charles C Thomas Publisher**:** pp 160-73.

Byers, T., S. Graham, T. Rzepka and J. Marshall (1985). "Lactation and breast cancer. Evidence for a negative association in premenopausal women." Am J Epidemiol **121**(5): 664-74.

Campos, L. S. (2004). "Neurospheres: insights into neural stem cell biology." J Neurosci Res **78**(6): 761-9.

Canfield, L. M., J. M. Hopkinson, A. F. Lima, B. Silva and C. Garza (1991). "Vitamin K in colostrum and mature human milk over the lactation period--a cross-sectional study." Am J Clin Nutr **53**(3): 730-5.

Cardoso, A. A., M. L. Li, P. Batard, A. Hatzfeld, E. L. Brown, J. P. Levesque, H. Sookdeo, B. Panterne, P. Sansilvestri, S. C. Clark and et al. (1993). "Release from quiescence of CD34+ CD38- human umbilical cord blood cells reveals their potentiality to engraft adults." Proc Natl Acad Sci U S A **90**(18): 8707-11.

Cariati, M., A. Naderi, J. P. Brown, M. J. Smalley, S. E. Pinder, C. Caldas and A. D. Purushotham (2008). "Alpha-6 integrin is necessary for the tumourigenicity of a

stem cell-like subpopulation within the MCF7 breast cancer cell line." Int J Cancer **122**(2): 298-304.

Cariati, M. and A. D. Purushotham (2008). "Stem cells and breast cancer." Histopathology **52**(1): 99-107.

Carow, C. E., G. Hangoc, S. H. Cooper, D. E. Williams and H. E. Broxmeyer (1991). "Mast cell growth factor (c-kit ligand) supports the growth of human multipotential progenitor cells with a high replating potential." Blood **78**(9): 2216-21.

Cervello, I., J. A. Martinez-Conejero, J. A. Horcajadas, A. Pellicer and C. Simon (2007). "Identification, characterization and co-localization of label-retaining cell population in mouse endometrium with typical undifferentiated markers." Hum Reprod **22**(1): 45-51.

Challen, G. A. and M. H. Little (2006). "A side order of stem cells: the SP phenotype." Stem Cells **24**(1): 3-12.

Chan, R. W. and C. E. Gargett (2006). "Identification of label-retaining cells in mouse endometrium." Stem Cells **24**(6): 1529-38.

Chapman, D. J. and R. Perez-Escamilla (1999). "Identification of risk factors for delayed onset of lactation." J Am Diet Assoc **99**(4): 450-4; quiz 455-6.

Chapman, D. J. and R. Perez-Escamilla (2000). "Maternal perception of the onset of lactation is a valid, public health indicator of lactogenesis stage II." J Nutr **130**(12): 2972-80.

Charrier, S., N. Boiret, M. Fouassier, J. Berger, C. Rapatel, P. Pigeon, G. Mareynat, J. Bonhomme, L. Camilleri and M. G. Berger (2002). "Normal human bone marrow CD34(+)CD133(+) cells contain primitive cells able to produce

different categories of colony-forming unit megakaryocytes in vitro." Exp Hematol **30**(9): 1051-60.

Check, E. (2002). "Cloning agenda 'skewed' by media frenzy." Nature **415**(6873): 722.

Chen, B. P., A. Galy, S. Kyoizumi, R. Namikawa, J. Scarborough, S. Webb, B. Ford, D. Z. Cen and S. C. Chen (1994). "Engraftment of human hematopoietic precursor cells with secondary transfer potential in SCID-hu mice." Blood **84**(8): 2497-505.

Chen, D. C., L. Nommsen-Rivers, K. G. Dewey and B. Lonnerdal (1998). "Stress during labor and delivery and early lactation performance." Am J Clin Nutr **68**(2): 335-44.

Chepko, G. and G. H. Smith (1997). "Three division-competent, structurally-distinct cell populations contribute to murine mammary epithelial renewal." Tissue Cell **29**(2): 239-53.

Civin, C. I., G. Almeida-Porada, M. J. Lee, J. Olweus, L. W. Terstappen and E. D. Zanjani (1996). "Sustained, retransplantable, multilineage engraftment of highly purified adult human bone marrow stem cells in vivo." Blood **88**(11): 4102-9.

Civin, C. I., L. C. Strauss, M. J. Fackler, T. M. Trischmann, J. M. Wiley and M. R. Loken (1990). "Positive stem cell selection--basic science." Prog Clin Biol Res **333**: 387-401; discussion 402.

Clark, A. D., H. G. Jorgensen, J. Mountford and T. L. Holyoake (2003). "Isolation and therapeutic potential of human haemopoietic stem cells." Cytotechnology **41**(2-3): 111-31.

Clarke, R. B., E. Anderson, A. Howell and C. S. Potten (2003). "Regulation of human breast epithelial stem cells." Cell Prolif **36 Suppl 1**: 45-58.

Clarke, R. B., K. Spence, E. Anderson, A. Howell, H. Okano and C. S. Potten (2005). "A putative human breast stem cell population is enriched for steroid receptor-positive cells." Dev Biol **277**(2): 443-56.

Clayton, H., I. Titley and M. Vivanco (2004). "Growth and differentiation of progenitor/stem cells derived from the human mammary gland." Exp Cell Res **297**(2): 444-60.

Clover, J., J. Oates and C. Edwards (1997). "Anti-cytokeratin 5/6: a positive marker for epithelioid mesothelioma." Histopathology **31**(2): 140-3.

Coleman, S., G. B. Silberstein and C. W. Daniel (1988). "Ductal morphogenesis in the mouse mammary gland: evidence supporting a role for epidermal growth factor." Dev Biol **127**(2): 304-15.

Coppa, G. V., O. Gabrielli, P. Pierani, C. Catassi, A. Carlucci and P. L. Giorgi (1993). "Changes in carbohydrate composition in human milk over 4 months of lactation." Pediatrics **91**(3): 637-41.

Coppa, G. V., P. Pierani, L. Zampini, I. Carloni, A. Carlucci and O. Gabrielli (1999). "Oligosaccharides in human milk during different phases of lactation." Acta Paediatr Suppl **88**(430): 89-94.

Cox, T. M., J. Mazurier, G. Spik, J. Montreuil and T. J. Peters (1979). "Iron binding proteins and influx of iron across the duodenal brush border. Evidence for specific lactotransferrin receptors in the human intestine." Biochim Biophys Acta **588**(1): 120-8.

Crago, S. S., S. J. Prince, T. G. Pretlow, J. R. McGhee and J. Mestecky (1979). "Human colostral cells. I. Separation and characterization." Clin Exp Immunol **38**(3): 585-97.

Craig, W., R. Kay, R. L. Cutler and P. M. Lansdorp (1993). "Expression of Thy-1 on human hematopoietic progenitor cells." J Exp Med **177**(5): 1331-42.

Cregan, M. D., Y. Fan, A. Appelbee, M. L. Brown, B. Klopcic, J. Koppen, L. R. Mitoulas, K. M. Piper, M. A. Choolani, Y. S. Chong and P. E. Hartmann (2007). "Identification of nestin-positive putative mammary stem cells in human breastmilk." Cell Tissue Res **329**(1): 129-36.

Cross, N. A., L. S. Hillman, S. H. Allen and G. F. Krause (1995). "Changes in bone mineral density and markers of bone remodeling during lactation and postweaning in women consuming high amounts of calcium." J Bone Miner Res **10**(9): 1312-20.

Cunha, G. R., P. Young, K. Christov, R. Guzman, S. Nandi, F. Talamantes and G. Thordarson (1995). "Mammary phenotypic expression induced in epidermal cells by embryonic mammary mesenchyme." Acta Anat (Basel) **152**(3): 195-204.

Cushing, A. H., J. M. Samet, W. E. Lambert, B. J. Skipper, W. C. Hunt, S. A. Young and L. C. McLaren (1998). "Breastfeeding reduces risk of respiratory illness in infants." Am J Epidemiol **147**(9): 863-70.

Dahlstrand, J., V. P. Collins and U. Lendahl (1992). "Expression of the class VI intermediate filament nestin in human central nervous system tumors." Cancer Res **52**(19): 5334-41.

Davies, B. R., R. Barraclough, M. P. Davies and P. S. Rudland (1993). "Production of the metastatic phenotype by DNA transfection in a rat mammary model." Cell Biol Int **17**(9): 871-9.

Davies, D. T., Holt, C. and Christie, W.W. (1983). The composition of milk. Biochemistry of Lactation. T. B. Mepham. Amsterdam, Elsevier Science Publisher**:** pp 71-117.

Davis, M. K., D. A. Savitz and B. I. Graubard (1988). "Infant feeding and childhood cancer." Lancet **2**(8607): 365-8.

De Sole, G., Y. Belay and B. Zegeye (1987). "Vitamin A deficiency in southern Ethiopia." Am J Clin Nutr **45**(4): 780-4.

de Wynter, E. A., D. Buck, C. Hart, R. Heywood, L. H. Coutinho, A. Clayton, J. A. Rafferty, D. Burt, G. Guenechea, J. A. Bueren, D. Gagen, L. J. Fairbairn, B. I. Lord and N. G. Testa (1998). "CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors." Stem Cells **16**(6): 387-96.

Deome, K. B., L. J. Faulkin, Jr., H. A. Bern and P. B. Blair (1959). "Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice." Cancer Res **19**(5): 515-20.

Dewey, K. G. (2001). "Maternal and fetal stress are associated with impaired lactogenesis in humans." J Nutr **131**(11): 3012S-5S.

Dewey, K. G., M. J. Heinig and L. A. Nommsen-Rivers (1995). "Differences in morbidity between breast-fed and formula-fed infants." J Pediatr **126**(5 Pt 1): 696-702.

Dewey, K. G., L. A. Nommsen-Rivers, M. J. Heinig and R. J. Cohen (2001). Lactogenesis and infant weight change in the first weeks of life. Integrating Population Outcomes, Biological Mechanisms, and Research Methods in the Study of Human Milk and Lactation. M. K. Davis, A. L. Wright, C. E. Issacs and L. Hanson. New York, Kluwer Academic/ Plenum Publishers**:** pp 159-166.

Dontu, G., W. M. Abdallah, J. M. Foley, K. W. Jackson, M. F. Clarke, M. J. Kawamura and M. S. Wicha (2003a). "In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells." Genes Dev **17**(10): 1253-70.

Dontu, G., M. Al-Hajj, W. M. Abdallah, M. F. Clarke and M. S. Wicha (2003b). "Stem cells in normal breast development and breast cancer." Cell Prolif **36 Suppl 1**: 59-72.

Dontu, G., K. W. Jackson, E. McNicholas, M. J. Kawamura, W. M. Abdallah and M. S. Wicha (2004). "Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells." Breast Cancer Res **6**(6): R605-15.

Dontu, G., S. Liu and M. S. Wicha (2005). "Stem cells in mammary development and carcinogenesis: implications for prevention and treatment." Stem Cell Rev **1**(3): 207-13.

Dontu, G. and M. S. Wicha (2005). "Survival of mammary stem cells in suspension culture: implications for stem cell biology and neoplasia." J Mammary Gland Biol Neoplasia **10**(1): 75-86.

Duffy, L. C., H. Faden, R. Wasielewski, J. Wolf and D. Krystofik (1997). "Exclusive breastfeeding protects against bacterial colonization and day care exposure to otitis media." Pediatrics **100**(4): E7.

Duncan, B., J. Ey, C. J. Holberg, A. L. Wright, F. D. Martinez and L. M. Taussig (1993). "Exclusive breast-feeding for at least 4 months protects against otitis media." Pediatrics **91**(5): 867-72.

Duncan, R. L., Jr. and W. P. McArthur (1981). "Lactoferrin-mediated modulation of mononuclear cell activities. I. Suppression of the murine in vitro primary antibody responses." Cell Immunol **63**(2): 308-20.

Eaker, S. S., T. S. Hawley, A. Ramezani and R. G. Hawley (2004). "Detection and enrichment of hematopoietic stem cells by side population phenotype." Methods Mol Biol **263**: 161-80.

Ebert, E. C. (1995). "Human intestinal intraepithelial lymphocytes have potent chemotactic activity." Gastroenterology **109**(4): 1154-9.

Ebner, K. E. and F. L. Schanbacher (1974). Biochemistry of lactose and related carbohydrates. Lactation: A Comprahensive Treatise. B. L. Larson and V. R. Smith. New York, Academic Press**:** pp 77-103.

Eirew, P., J. Stingl, A. Raouf, G. Turashvili, S. Aparicio, J. T. Emerman and C. J. Eaves (2008). "A method for quantifying normal human mammary epithelial stem cells with in vivo regenerative ability." Nat Med **14**(12): 1384-9.

Ellis, L. A., A. M. Mastro and M. F. Picciano (1997). "Do milk-borne cytokines and hormones influence neonatal immune cell function?" J Nutr **127**(5 Suppl): 985S-988S.

Ellis, L. A. and M. F. Picciano (1992). "Milk-borne hormones: regulators of development in neonates." Nutr Today **27**: 6-14.

Ellison, R. T., 3rd and T. J. Giehl (1991). "Killing of gram-negative bacteria by lactoferrin and lysozyme." J Clin Invest **88**(4): 1080-91.

Emodi, G. and M. Just (1974). "Interferon production by lymphocytes in human milk." Scand J Immunol **3**(2): 157-60.

Enger, S. M., R. K. Ross, B. Henderson and L. Bernstein (1997). "Breastfeeding history, pregnancy experience and risk of breast cancer." Br J Cancer **76**(1): 118-23.

Fata, J. E., Z. Werb and M. J. Bissell (2004). "Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes." Breast Cancer Res **6**(1): 1-11.

Fina, L., H. V. Molgaard, D. Robertson, N. J. Bradley, P. Monaghan, D. Delia, D. R. Sutherland, M. A. Baker and M. F. Greaves (1990). "Expression of the CD34 gene in vascular endothelial cells." Blood **75**(12): 2417-26.

Forbes, S. J., R. Poulsom and N. A. Wright (2002). "Hepatic and renal differentiation from blood-borne stem cells." Gene Ther **9**(10): 625-30.

Franke, W. W., B. Appelhans, E. Schmid, C. Freudenstein, M. Osborn and K. Weber (1979). "Identification and characterization of epithelial cells in mammalian tissues by immunofluorescence microscopy using antibodies to prekeratin." Differentiation **15**(1): 7-25.

Franke, W. W., E. Schmid, C. Freudenstein, B. Appelhans, M. Osborn, K. Weber and T. W. Keenan (1980). "Intermediate-sized filaments of the prekeratin type in myoepithelial cells." J Cell Biol **84**(3): 633-54.

Fuchs, E., T. Tumbar and G. Guasch (2004). "Socializing with the neighbors: stem cells and their niche." Cell **116**(6): 769-78.

Fujihashi, K., J. R. McGhee, C. Lue, K. W. Beagley, T. Taga, T. Hirano, T. Kishimoto, J. Mestecky and H. Kiyono (1991). "Human appendix B cells naturally express receptors for and respond to interleukin 6 with selective IgA1 and IgA2 synthesis." J Clin Invest **88**(1): 248-52.

Gaffney, E. V. (1982). "A cell line (HBL-100) established from human breast milk." Cell Tissue Res **227**(3): 563-8.

Gage, F. H. (2000). "Mammalian neural stem cells." Science **287**(5457): 1433-8.

Gala, R. R. (1991). "Prolactin and growth hormone in the regulation of the immune system." Proc Soc Exp Biol Med **198**(1): 513-27.

Gallacher, L., B. Murdoch, D. M. Wu, F. N. Karanu, M. Keeney and M. Bhatia (2000). "Isolation and characterization of human CD34(-)Lin(-) and CD34(+)Lin(-) hematopoietic stem cells using cell surface markers AC133 and CD7." Blood **95**(9): 2813-20.

Gargett, C. E. (2007). "Uterine stem cells: what is the evidence?" Hum Reprod Update **13**(1): 87-101.

Garofalo, R., S. Chheda, F. Mei, K. H. Palkowetz, H. E. Rudloff, F. C. Schmalstieg, D. K. Rassin and A. S. Goldman (1995). "Interleukin-10 in human milk." Pediatr Res **37**(4 Pt 1): 444-9.

Garofalo, R. P. and A. S. Goldman (1998). "Cytokines, chemokines, and colony-stimulating factors in human milk: the 1997 update." Biol Neonate **74**(2): 134-42.

Garofalo, R. P. and A. S. Goldman (1999). "Expression of functional immunomodulatory and anti-inflammatory factors in human milk." Clin Perinatol **26**(2): 361-77.

Genzel-Boroviczeny, O., J. Wahle and B. Koletzko (1997). "Fatty acid composition of human milk during the 1st month after term and preterm delivery." Eur J Pediatr **156**(2): 142-7.

Gerstein, H. C. (1994). "Cow's milk exposure and type I diabetes mellitus. A critical overview of the clinical literature." Diabetes Care **17**(1): 13-9.

Giorno, R. (1985). "Immunohistochemical analysis of the distribution of vimentin in human peripheral lymphoid tissues." Anat Rec **211**(1): 43-7.

Goldblum, R. M., R. J. Schanler, C. Garza and A. S. Goldman (1989). "Human milk feeding enhances the urinary excretion of immunologic factors in low birth weight infants." Pediatr Res **25**(2): 184-8.

Goldman, A. S., S. Chheda and R. Garofalo (1998). "Evolution of immunologic functions of the mammary gland and the postnatal development of immunity." Pediatr Res **43**(2): 155-62.

Goldman, A. S. and R. M. Goldblum (1989). Immunoglobulins in human milk. Protein and Non-protein Nitrogen in Human Milk. S. A. Atkinson and B. Lonnerdal. Boca Raton, CRC Press: pp 43-51.

Goodell, M. A., K. Brose, G. Paradis, A. S. Conner and R. C. Mulligan (1996). "Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo." J Exp Med **183**(4): 1797-806.

Goodell, M. A., M. Rosenzweig, H. Kim, D. F. Marks, M. DeMaria, G. Paradis, S. A. Grupp, C. A. Sieff, R. C. Mulligan and R. P. Johnson (1997). "Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species." Nat Med **3**(12): 1337-45.

Grant, M. B., W. S. May, S. Caballero, G. A. Brown, S. M. Guthrie, R. N. Mames, B. J. Byrne, T. Vaught, P. E. Spoerri, A. B. Peck and E. W. Scott (2002). "Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization." Nat Med **8**(6): 607-12.

Gregory, C. A., H. Singh, A. S. Perry and D. J. Prockop (2003). "The Wnt signaling inhibitor dickkopf-1 is required for reentry into the cell cycle of human adult stem cells from bone marrow." J Biol Chem **278**(30): 28067-78.

Grosvenor, C. E., M. F. Picciano and C. R. Baumrucker (1993). "Hormones and growth factors in milk." Endocr Rev **14**(6): 710-28.

Gudjonsson, T., L. Ronnov-Jessen, R. Villadsen, F. Rank, M. J. Bissell and O. W. Petersen (2002a). "Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition." J Cell Sci **115**(Pt 1): 39-50.

Gudjonsson, T., R. Villadsen, H. L. Nielsen, L. Ronnov-Jessen, M. J. Bissell and O. W. Petersen (2002b). "Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties." Genes Dev **16**(6): 693-706.

Gunji, Y., M. Nakamura, H. Osawa, K. Nagayoshi, H. Nakauchi, Y. Miura, M. Yanagisawa and T. Suda (1993). "Human primitive hematopoietic progenitor cells are more enriched in KITlow cells than in KIThigh cells." Blood **82**(11): 3283-9.

Gussoni, E., Y. Soneoka, C. D. Strickland, E. A. Buzney, M. K. Khan, A. F. Flint, L. M. Kunkel and R. C. Mulligan (1999). "Dystrophin expression in the mdx mouse restored by stem cell transplantation." Nature **401**(6751): 390-4.

Gusterson, B. A., P. Monaghan, R. Mahendran, J. Ellis and M. J. O'Hare (1986). "Identification of myoepithelial cells in human and rat breasts by anti-common acute lymphoblastic leukemia antigen antibody A12." J Natl Cancer Inst **77**(2): 343-9.

Guyette, W. A., R. J. Matusik and J. M. Rosen (1979). "Prolactin-mediated transcriptional and post-transcriptional control of casein gene expression." Cell **17**(4): 1013-23.

Hamosh, M. (1995). Enzymes in human milk. Handbook of Milk Composition. R. G. Jensen. San Diego, Academic Press**:** pp 388-427.

Hamosh, M. (2001). "Bioactive factors in human milk." Pediatr Clin North Am **48**(1): 69-86.

Handgretinger, R., P. R. Gordon, T. Leimig, X. Chen, H. J. Buhring, D. Niethammer and S. Kuci (2003). "Biology and plasticity of CD133+ hematopoietic stem cells." Ann N Y Acad Sci **996**: 141-51.

Hao, Q. L., A. J. Shah, F. T. Thiemann, E. M. Smogorzewska and G. M. Crooks (1995). "A functional comparison of CD34 + CD38- cells in cord blood and bone marrow." Blood **86**(10): 3745-53.

Harrison, D. E. (1980). "Competitive repopulation: a new assay for long-term stem cell functional capacity." Blood **55**(1): 77-81.

Harrison, D. E., C. T. Jordan, R. K. Zhong and C. M. Astle (1993). "Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations." Exp Hematol **21**(2): 206-19.

Hartmann, P. and M. Cregan (2001). "Lactogenesis and the effects of insulin-dependent diabetes mellitus and prematurity." J Nutr **131**(11): 3016S-20S.

Hartmann, P. E. (1973). "Changes in the composition and yield of the mammary secretion of cows during the initiation of lactation." J Endocrinol **59**(2): 231-47.

Hawley, R. G., A. Rameszani and T. S. Hawley (2006). Hematopoietic stem cells. Methods in Enzymology. I. Klimanskaya and R. Lanza. San Diego, Academic Press. **419:** pp 149-179.

Hayashi, R., M. Yamato, T. Saito, T. Oshima, T. Okano, Y. Tano and K. Nishida (2008). "Enrichment of corneal epithelial stem/progenitor cells using cell surface markers, integrin [alpha]6 and CD71." Biochemical and Biophysical Research Communications **367**(2): 256-263.

He, J. and P. Furmanski (1995). "Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA." Nature **373**(6516): 721-4.

Henry, M. D., A. A. Triplett, K. B. Oh, G. H. Smith and K. U. Wagner (2004). "Parity-induced mammary epithelial cells facilitate tumorigenesis in MMTV-neu transgenic mice." Oncogene **23**(41): 6980-5.

Hernell, O., L. Blackberg and S. Bernback (1989). "Digestion of human milk fat in early infancy." Acta Paediatr Scand Suppl **351**: 57-62.

Hess, D. A., L. Wirthlin, T. P. Craft, P. E. Herrbrich, S. A. Hohm, R. Lahey, W. C. Eades, M. H. Creer and J. A. Nolta (2006). "Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells." Blood **107**(5): 2162-9.

Hida, N., N. Nishiyama, S. Miyoshi, S. Kira, K. Segawa, T. Uyama, T. Mori, K. Miyado, Y. Ikegami, C. Cui, T. Kiyono, S. Kyo, T. Shimizu, T. Okano, M. Sakamoto, S. Ogawa and A. Umezawa (2008). "Novel cardiac precursor-like cells from human menstrual blood-derived mesenchymal cells." Stem Cells **26**(7): 1695-704.

Hill, B., E. Rozler, M. Travis, S. Chen, A. Zannetino, P. Simmons, A. Galy, B. Chen and R. Hoffman (1996). "High-level expression of a novel epitope of CD59 identifies a subset of CD34+ bone marrow cells highly enriched for pluripotent stem cells." Exp Hematol **24**(8): 936-43.

Hilson, J. A., K. M. Rasmussen and C. L. Kjolhede (1997). "Maternal obesity and breast-feeding success in a rural population of white women." Am J Clin Nutr **66**(6): 1371-8.

Ho, F. C., R. L. Wong and J. W. Lawton (1979). "Human colostral and breast milk cells. A light and electron microscopic study." Acta Paediatr Scand **68**(3): 389-96.

Hobbs, A. A., D. A. Richards, D. J. Kessler and J. M. Rosen (1982). "Complex hormonal regulation of rat casein gene expression." J Biol Chem **257**(7): 3598-605.

Horwitz, E. M., D. J. Prockop, L. A. Fitzpatrick, W. W. Koo, P. L. Gordon, M. Neel, M. Sussman, P. Orchard, J. C. Marx, R. E. Pyeritz and M. K. Brenner (1999).

"Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta." Nat Med **5**(3): 309-13.

Hovey, R. C. and J. F. Trott (2004). "Morphogenesis of mammary gland development." Adv Exp Med Biol **554**: 219-28.

Hovey, R. C., J. F. Trott and B. K. Vonderhaar (2002). "Establishing a framework for the functional mammary gland: from endocrinology to morphology." J Mammary Gland Biol Neoplasia **7**(1): 17-38.

Howard, B. A. and B. A. Gusterson (2000). "Human breast development." J Mammary Gland Biol Neoplasia **5**(2): 119-37.

Howell, R. R., P. A. Palma, C. R. West, R. M. Caprioli and W. E. Seifert (1986). Trace elements in human milk: differences over time and between population groups. Human Milk in Infant Nutrition and Healt. R. R. Howell, F. H. Morriss and L. K. Pickering. Springfield, Charles C. Thomas Publisher**:** pp 28-50.

Howie, P. W., J. S. Forsyth, S. A. Ogston, A. Clark and C. D. Florey (1990). "Protective effect of breast feeding against infection." Bmj **300**(6716): 11-6.

Huang, S. and L. W. Terstappen (1994). "Lymphoid and myeloid differentiation of single human CD34+, HLA-DR+, CD38- hematopoietic stem cells." Blood **83**(6): 1515-26.

Humphries, R. K., A. C. Eaves and C. J. Eaves (1981). "Self-renewal of hemopoietic stem cells during mixed colony formation in vitro." Proc Natl Acad Sci U S A **78**(6): 3629-33.

Hurst, N. M. (2007). "Recognizing and treating delayed or failed lactogenesis II." J Midwifery Womens Health **52**(6): 588-94.

Institute of Medicine. (1991). "Nutrition during lactation." Washington, DC, National Academy Press.

Isaacs, C. E., T. Pascal, C. E. Wright and G. E. Gaull (1984). "Sulfhydryl oxidase in human milk: stability of milk enzymes in the gastrointestinal tract." Pediatr Res **18**(6): 532-5.

Islam, M. O., Y. Kanemura, J. Tajria, H. Mori, S. Kobayashi, T. Shofuda, J. Miyake, M. Hara, M. Yamasaki and H. Okano (2005). "Characterization of ABC transporter ABCB1 expressed in human neural stem/progenitor cells." FEBS Lett **579**(17): 3473-80.

Jain, L., D. Vidyasagar, M. Xanthou, V. Ghai, S. Shimada and M. Blend (1989). "In vivo distribution of human milk leucocytes after ingestion by newborn baboons." Arch Dis Child **64**(7 Spec No): 930-3.

Jensen, R. G. (1989). The Lipids of Human Milk. Boca Raton, CRC Press.

Jensen, R. G. (1996). "The lipids in human milk." Prog Lipid Res **35**(1): 53-92.

Joshi, K., J. A. Smith, N. Perusinghe and P. Monoghan (1986). "Cell proliferation in the human mammary epithelium. Differential contribution by epithelial and myoepithelial cells." Am J Pathol **124**(2): 199-206.

Kabos, P., M. Ehtesham, A. Kabosova, K. L. Black and J. S. Yu (2002). "Generation of neural progenitor cells from whole adult bone marrow." Exp Neurol **178**(2): 288-93.

Kalirai, H. and R. B. Clarke (2006). "Human breast epithelial stem cells and their regulation." J Pathol **208**(1): 7-16.

Katsouyanni, K., D. Trichopoulos, P. Boyle, E. Xirouchaki, A. Trichopoulou, B. Lisseos, S. Vasilaros and B. MacMahon (1986). "Diet and breast cancer: a case-control study in Greece." Int J Cancer **38**(6): 815-20.

Kaufman, C. L. and S. T. Ildstad (1999). "Leukodystrophy and bone marrow transplantation: role of mixed hematopoietic chimerism." Neurochem Res **24**(4): 537-49.

Kawashima, I., E. D. Zanjani, G. Almaida-Porada, A. W. Flake, H. Zeng and M. Ogawa (1996). "CD34+ human marrow cells that express low levels of Kit protein are enriched for long-term marrow-engrafting cells." Blood **87**(10): 4136-42.

Kennedy, K. I. (1994). "Effects of breastfeeding on women's health." Int J Gynaecol Obstet **47 Suppl**: S11-20; discussion S20-1.

Kenney, N. J., A. Bowman, K. S. Korach, J. C. Barrett and D. S. Salomon (2003). "Effect of exogenous epidermal-like growth factors on mammary gland development and differentiation in the estrogen receptor-alpha knockout (ERKO) mouse." Breast Cancer Res Treat **79**(2): 161-73.

Kerr, J. F., A. H. Wyllie and A. R. Currie (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**(4): 239-57.

Kerre, T. C., G. De Smet, M. De Smedt, F. Offner, J. De Bosscher, J. Plum and B. Vandekerckhove (2001). "Both CD34+38+ and CD34+38- cells home specifically to the bone marrow of NOD/LtSZ scid/scid mice but show different kinetics in expansion." J Immunol **167**(7): 3692-8.

Knight, C. H. (1995). The environment of the mammary secretory cell. Intercellular signalling in the mammary gland. C. J. Wilde, M. Peaker and C. H. Knight. New York, Plenum Press: pp 1-12.

Kobari, L., M. C. Giarratana, F. Pflumio, B. Izac, L. Coulombel and L. Douay (2001). "CD133+ cell selection is an alternative to CD34+ cell selection for ex vivo

expansion of hematopoietic stem cells." J Hematother Stem Cell Res **10**(2): 273-81.

Koldovsky, O. (1995). "Hormones in milk." Vitam Horm **50**: 77-149.

Koletzko, B. and M. Braun (1991). "Arachidonic acid and early human growth: is there a relation?" Ann Nutr Metab **35**(3): 128-31.

Koletzko, S., P. Sherman, M. Corey, A. Griffiths and C. Smith (1989). "Role of infant feeding practices in development of Crohn's disease in childhood." Bmj **298**(6688): 1617-8.

Kordon, E. C. and G. H. Smith (1998). "An entire functional mammary gland may comprise the progeny from a single cell." Development **125**(10): 1921-30.

Krause, S., M. V. Maffini, A. M. Soto and C. Sonnenschein (2008). "A novel 3D in vitro culture model to study stromal-epithelial interactions in the mammary gland." Tissue Eng Part C Methods **14**(3): 261-71.

Krebs, N. F., C. J. Reidinger, A. D. Robertson and M. Brenner (1997). "Bone mineral density changes during lactation: maternal, dietary, and biochemical correlates." Am J Clin Nutr **65**(6): 1738-46.

Krivit, W., C. Peters and E. G. Shapiro (1999). "Bone marrow transplantation as effective treatment of central nervous system disease in globoid cell leukodystrophy, metachromatic leukodystrophy, adrenoleukodystrophy, mannosidosis, fucosidosis, aspartylglucosaminuria, Hurler, Maroteaux-Lamy, and Sly syndromes, and Gaucher disease type III." Curr Opin Neurol **12**(2): 167-76.

Kuhn, N. J. (1983). The biochemistry of lactogenesis. Biochemistry of Lactation. T. B. Mepham. Amsterdam, Elsevier**:** pp 351-380.

Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky and W. Muller (1993). "Interleukin-10-deficient mice develop chronic enterocolitis." Cell **75**(2): 263-74.

Kulkarni, A. D., W. C. Fanslow, F. B. Rudolph and C. T. Van Buren (1986). "Effect of dietary nucleotides on response to bacterial infections." JPEN J Parenter Enteral Nutr **10**(2): 169-71.

Kulski, J. K., P. E. Hartmann, J. D. Martin and M. Smith (1978). "Effects of bromocriptine mesylate on the composition of the mammary secretion in non-breast-feeding women." Obstet Gynecol **52**(1): 38-42.

Kunz, C., M. Rodriguez-Palmero, B. Koletzko and R. Jensen (1999). "Nutritional and biochemical properties of human milk, Part I: General aspects, proteins, and carbohydrates." Clin Perinatol **26**(2): 307-33.

Labbok, M. H. (2001). "Effects of breastfeeding on the mother." Pediatr Clin North Am **48**(1): 143-58.

Laegreid, A., A. B. Otnaess and J. Fuglesang (1986). "Human and bovine milk: comparison of ganglioside composition and enterotoxin-inhibitory activity." Pediatr Res **20**(5): 416-21.

Lagasse, E., H. Connors, M. Al-Dhalimy, M. Reitsma, M. Dohse, L. Osborne, X. Wang, M. Finegold, I. L. Weissman and M. Grompe (2000). "Purified hematopoietic stem cells can differentiate into hepatocytes in vivo." Nat Med **6**(11): 1229-34.

Lajtha, L. G. (1979). "Stem cell concepts." Differentiation **14**(1-2): 23-34.

Lammi-Keefe, C. J. (1995). Vitamins D and E in human milk. Handbook of Milk Composition. R. Jensen. New York, Academic Press**:** pp 706-717.

Lang, P., P. Bader, M. Schumm, T. Feuchtinger, H. Einsele, M. Fuhrer, C. Weinstock, R. Handgretinger, S. Kuci, D. Martin, D. Niethammer and J. Greil (2004).

"Transplantation of a combination of CD133+ and CD34+ selected progenitor cells from alternative donors." Br J Haematol **124**(1): 72-9.

Lansdorp, P. M., W. Dragowska and H. Mayani (1993). "Ontogeny-related changes in proliferative potential of human hematopoietic cells." J Exp Med **178**(3): 787-91.

Lanting, C. I., V. Fidler, M. Huisman, B. C. Touwen and E. R. Boersma (1994). "Neurological differences between 9-year-old children fed breast-milk or formula-milk as babies." Lancet **344**(8933): 1319-22.

Larochelle, A., J. Vormoor, H. Hanenberg, J. C. Wang, M. Bhatia, T. Lapidot, T. Moritz, B. Murdoch, X. L. Xiao, I. Kato, D. A. Williams and J. E. Dick (1996). "Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy." Nat Med **2**(12): 1329-37.

Layde, P. M., L. A. Webster, A. L. Baughman, P. A. Wingo, G. L. Rubin and H. W. Ory (1989). "The independent associations of parity, age at first full term pregnancy, and duration of breastfeeding with the risk of breast cancer. Cancer and Steroid Hormone Study Group." J Clin Epidemiol **42**(10): 963-73.

Leemhuis, T., M. C. Yoder, S. Grigsby, B. Aguero, P. Eder and E. F. Srour (1996). "Isolation of primitive human bone marrow hematopoietic progenitor cells using Hoechst 33342 and Rhodamine 123." Exp Hematol **24**(10): 1215-24.

Lendahl, U., L. B. Zimmerman and R. D. McKay (1990). "CNS stem cells express a new class of intermediate filament protein." Cell **60**(4): 585-95.

Lewis-Jones, D. I., M. S. Lewis-Jones, R. C. Connolly, D. C. Lloyd and C. R. West (1985). "Sequential changes in the antimicrobial protein concentrations in human milk during lactation and its relevance to banked human milk." Pediatr Res **19**(6): 561-5.

Li, H., P. Cherukuri, N. Li, V. Cowling, M. Spinella, M. Cole, A. K. Godwin, W. Wells and J. DiRenzo (2007). "Nestin is expressed in the basal/myoepithelial layer of the mammary gland and is a selective marker of basal epithelial breast tumors." Cancer Res **67**(2): 501-10.

Lindberg, T., K. Ohlsson and B. Westrom (1982). "Protease inhibitors and their relation to protease activity in human milk." Pediatr Res **16**(6): 479-83.

Link, H., L. Arseniev, O. Bahre, J. G. Kadar, H. Diedrich and H. Poliwoda (1996). "Transplantation of allogeneic CD34+ blood cells." Blood **87**(11): 4903-9.

Linzell, J. L. (1952). "The silver staining of myoepithelial cells, particularly in the mammary gland, and their relation to the ejection of milk." J Anat **86**(1): 49-57.

Lord, B. I. (1997). Biology of the haemopoietic stem cell. Stem Cells. C. S. Potten. San Diego, Academic Press**:** pp 401-422.

Lu, L., M. Xiao, R. N. Shen, S. Grigsby and H. E. Broxmeyer (1993). "Enrichment, characterization, and responsiveness of single primitive CD34 human umbilical cord blood hematopoietic progenitors with high proliferative and replating potential." Blood **81**(1): 41-8.

Lu, X., S. V. Baudouin, J. I. Gillespie, J. J. Anderson and A. M. Dickinson (2007). "A comparison of CFU-GM, BFU-E and endothelial progenitor cells using ex vivo expansion of selected cord blood CD133(+) and CD34(+) cells." Cytotherapy **9**(3): 292-300.

Lucas, A., R. Morley, T. J. Cole, G. Lister and C. Leeson-Payne (1992). "Breast milk and subsequent intelligence quotient in children born preterm." Lancet **339**(8788): 261-4.

Lucy, J. A. (1972). "Functional and structural aspects of biological membranes: a suggested structural role for vitamin E in the control of membrane permeability and stability." Ann N Y Acad Sci **203**: 4-11.

Lund, L. R., J. Romer, N. Thomasset, H. Solberg, C. Pyke, M. J. Bissell, K. Dano and Z. Werb (1996). "Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways." Development **122**(1): 181-93.

Madsen, K. L., S. A. Lewis, M. M. Tavernini, J. Hibbard and R. N. Fedorak (1997). "Interleukin 10 prevents cytokine-induced disruption of T84 monolayer barrier integrity and limits chloride secretion." Gastroenterology **113**(1): 151-9.

Markl, J. and N. Schechter (1998). "Fish intermediate filament proteins in structure, evolution, and function." Subcell Biochem **31**: 1-33.

Marshman, E., C. Booth and C. S. Potten (2002). "The intestinal epithelial stem cell." Bioessays **24**(1): 91-8.

Mata, L. J. and R. G. Wyatt (1971). "The uniqueness of human milk. Host resistance to infection." Am J Clin Nutr **24**(8): 976-86.

Mather, I. H. and T. W. Keenan (1983). Function of endomembrane in secretion of organic milk constituents. Biochemistry of Lactation. T. B. Mepham. Amsterdam, Elsevier**:** pp 231-283.

Mattsby-Baltzer, I., A. Roseanu, C. Motas, J. Elverfors, I. Engberg and L. A. Hanson (1996). "Lactoferrin or a fragment thereof inhibits the endotoxin-induced interleukin-6 response in human monocytic cells." Pediatr Res **40**(2): 257-62.

Maxson, R. T., R. J. Jackson and S. D. Smith (1995). "The protective role of enteral IgA supplementation in neonatal gut origin sepsis." J Pediatr Surg **30**(2): 231-3; discussion 233-4.

McAlister, I., N. S. Wolf, M. E. Pietrzyk, P. S. Rabinovitch, G. Priestley and B. Jaeger (1990). "Transplantation of hematopoietic stem cells obtained by a combined dye method fractionation of murine bone marrow." Blood **75**(6): 1240-6.

McCredie, M., C. Paul, D. C. Skegg and S. Williams (1998). "Reproductive factors and breast cancer in New Zealand." Int J Cancer **76**(2): 182-8.

McCulloch, E. A. and J. E. Till (2005). "Perspectives on the properties of stem cells." Nat Med **11**(10): 1026-8.

McGregor, J. A. and L. J. Rogo (2006). "Breast milk: an unappreciated source of stem cells." J Hum Lact **22**(3): 270-1.

McTiernan, A. and D. B. Thomas (1986). "Evidence for a protective effect of lactation on risk of breast cancer in young women. Results from a case-control study." Am J Epidemiol **124**(3): 353-8.

Mepham, T. B. (1983). Physiological aspects of lactation. Biochemistry of Lactation. T. B. Mepham. Amsterdam, Elsevier**:** pp 3-28.

Mezey, E., K. J. Chandross, G. Harta, R. A. Maki and S. R. McKercher (2000). "Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow." Science **290**(5497): 1779-82.

Michalopoulos, G. K. and M. C. DeFrances (1997). "Liver regeneration." Science **276**(5309): 60-6.

Mischke, D. and G. Wild (1987). "Polymorphic keratins in human epidermis." J Invest Dermatol **88**(2): 191-7.

Mohr, J. A. (1973). "The possible induction and-or acquisition of cellular hypersensitivity associated with ingestion of colostrum." J Pediatr **82**(6): 1062-4.

Moore, K. W., A. O'Garra, R. de Waal Malefyt, P. Vieira and T. R. Mosmann (1993). "Interleukin-10." Annu Rev Immunol **11**: 165-90.

Morgan, O. S., J. Bankay and G. A. Quash (1975). "The effect of lactoferrin, an iron-binding protein on complement activity." West Indian Med J **24**(1): 46-54.

Morriss, F. H. (1986). Growth factors in milk. . Human Milk in Infant Nutrition and Health. . R. R. Howell, F. H. Morriss and L. K. Pickering. Springfield, Charles C Thomas Publisher.

Morroni, M., A. Giordano, M. C. Zingaretti, R. Boiani, R. De Matteis, B. B. Kahn, E. Nisoli, C. Tonello, C. Pisoschi, M. M. Luchetti, M. Marelli and S. Cinti (2004). "Reversible transdifferentiation of secretory epithelial cells into adipocytes in the mammary gland." Proc Natl Acad Sci U S A **101**(48): 16801-6.

Morton, J. A. (1994). "The clinical usefulness of breast milk sodium in the assessment of lactogenesis." Pediatrics **93**(5): 802-6.

Murillo, G. J. and A. S. Goldman (1970). "The cells of human colostrum. II. Synthesis of IgA and Beta1c." Pediatr Res **4**(1): 71-5.

Murray, L., B. Chen, A. Galy, S. Chen, R. Tushinski, N. Uchida, R. Negrin, G. Tricot, S. Jagannath, D. Vesole and et al. (1995). "Enrichment of human hematopoietic stem cell activity in the CD34+Thy-1+Lin- subpopulation from mobilized peripheral blood." Blood **85**(2): 368-78.

Musina, R. A., A. V. Belyavski, O. V. Tarusova, E. V. Solovyova and G. T. Sukhikh (2008). "Endometrial mesenchymal stem cells isolated from the menstrual blood." Bull Exp Biol Med **145**(4): 539-43.

Nair, M. P., S. A. Schwartz, H. B. Slade, M. Z. Johnson, J. F. Quebbeman and A. E. Beer (1985). "Comparison of the cellular cytotoxic activities of colostral lymphocytes and maternal peripheral blood lymphocytes." J Reprod Immunol **7**(3): 199-213.

Naylor, C. S., E. Jaworska, K. Branson, M. J. Embleton and R. Chopra (2005). "Side population/ABCG2-positive cells represent a heterogeneous group of haemopoietic cells: implications for the use of adult stem cells in transplantation and plasticity protocols." Bone Marrow Transplant **35**(4): 353-60.

Neifert, M. R., S. L. McDonough and M. C. Neville (1981). "Failure of lactogenesis associated with placental retention." Am J Obstet Gynecol **140**(4): 477-8.

Neubauer, S. H., A. M. Ferris, C. G. Chase, J. Fanelli, C. A. Thompson, C. J. Lammi-Keefe, R. M. Clark, R. G. Jensen, R. B. Bendel and K. W. Green (1993). "Delayed lactogenesis in women with insulin-dependent diabetes mellitus." Am J Clin Nutr **58**(1): 54-60.

Neville, M. C. (1999). "Physiology of lactation." Clin Perinatol **26**(2): 251-79.

Neville, M. C., J. C. Allen, P. C. Archer, C. E. Casey, J. Seacat, R. P. Keller, V. Lutes, J. Rasbach and M. Neifert (1991). "Studies in human lactation: milk volume and nutrient composition during weaning and lactogenesis." Am J Clin Nutr **54**(1): 81-92.

Neville, M. C., R. Keller, J. Seacat, V. Lutes, M. Neifert, C. Casey, J. Allen and P. Archer (1988). "Studies in human lactation: milk volumes in lactating women during the onset of lactation and full lactation." Am J Clin Nutr **48**(6): 1375-86.

Neville, M. C. and J. Morton (2001). "Physiology and endocrine changes underlying human lactogenesis II." J Nutr **131**(11): 3005S-8S.

Neville, M. C., J. Morton and S. Umemura (2001). "Lactogenesis. The transition from pregnancy to lactation." Pediatr Clin North Am **48**(1): 35-52.

Newcomb, P. A., B. E. Storer, M. P. Longnecker, R. Mittendorf, E. R. Greenberg, R. W. Clapp, K. P. Burke, W. C. Willett and B. MacMahon (1994). "Lactation and a reduced risk of premenopausal breast cancer." N Engl J Med **330**(2): 81-7.

Nimgaonkar, M. T., R. A. Roscoe, J. Persichetti, W. B. Rybka, A. Winkelstein and E. D. Ball (1995). "A unique population of CD34+ cells in cord blood." Stem Cells **13**(2): 158-66.

Nolta, J. A. and D. B. Kohn (1997). Haemapoietic stem cells for gene therapy. Stem Cells. C. S. Potten. San Diego, Academic Press**:** pp 447-462.

O'Donoghue, K., J. Chan, J. de la Fuente, N. Kennea, A. Sandison, J. R. Anderson, I. A. Roberts and N. M. Fisk (2004). "Microchimerism in female bone marrow and bone decades after fetal mesenchymal stem-cell trafficking in pregnancy." Lancet **364**(9429): 179-82.

O'Donoghue, K., H. A. Sultan, F. A. Al-Allaf, J. R. Anderson, J. Wyatt-Ashmead and N. M. Fisk (2008). "Microchimeric fetal cells cluster at sites of tissue injury in lung decades after pregnancy." Reprod Biomed Online **16**(3): 382-90.

Okano, H., H. Kawahara, M. Toriya, K. Nakao, S. Shibata and T. Imai (2005). "Function of RNA-binding protein Musashi-1 in stem cells." Exp Cell Res **306**(2): 349-56.

Ormerod, E. J. and P. S. Rudland (1986). "Regeneration of mammary glands in vivo from isolated mammary ducts." J Embryol Exp Morphol **96**: 229-43.

Owen, M. J., C. D. Baldwin, P. R. Swank, A. K. Pannu, D. L. Johnson and V. M. Howie (1993). "Relation of infant feeding practices, cigarette smoke exposure, and group child care to the onset and duration of otitis media with effusion in the first two years of life." J Pediatr **123**(5): 702-11.

Packard, V. S. (1982). Human Milk and Infant Formula. New York, Academic Press.

Parmely, M. J., D. B. Reath, A. E. Beer and R. E. Billingham (1977). "Cellular immune responses of human milk T lymphocytes to certain environmental antigens." Transplant Proc **9**(2): 1477-83.

Patton, S., G. E. Huston, P. A. Montgomery and R. V. Josephson (1986). Approaches to the study of colostrum-the onset of lactation. Human Lactation 2: Maternal and Environmental Factors. M. Hamosh and A. S. Goldman. New York, Plenum**:** pp 231-240.

Peaker, M. and C. J. Wilde (1996). "Feedback control of milk secretion from milk." J Mammary Gland Biol Neoplasia **1**(3): 307-15.

Pechoux, C., T. Gudjonsson, L. Ronnov-Jessen, M. J. Bissell and O. W. Petersen (1999). "Human mammary luminal epithelial cells contain progenitors to myoepithelial cells." Dev Biol **206**(1): 88-99.

Petersen, B. E., W. C. Bowen, K. D. Patrene, W. M. Mars, A. K. Sullivan, N. Murase, S. S. Boggs, J. S. Greenberger and J. P. Goff (1999). "Bone marrow as a potential source of hepatic oval cells." Science **284**(5417): 1168-70.

Petersen, O. W., L. Ronnov-Jessen, A. R. Howlett and M. J. Bissell (1992). "Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells." Proc Natl Acad Sci U S A **89**(19): 9064-8.

Picciano, M. F. and H. A. Guthrie (1976). "Copper, iron, and zinc contents of mature human milk." Am J Clin Nutr **29**(3): 242-54.

Piechaczek, C. (2001). "Cd133." J Biol Regul Homeost Agents **15**(1): 101-2.

Pisacane, A., L. Graziano, G. Mazzarella, B. Scarpellino and G. Zona (1992). "Breast-feeding and urinary tract infection." J Pediatr **120**(1): 87-9.

Pitelka, D. R., B. N. Taggart and S. T. Hamamoto (1983). "Effects of extracellular calcium depletion on membrane topography and occluding junctions of mammary epithelial cells in culture." J Cell Biol **96**(3): 613-24.

Pitt, J., B. Barlow and W. C. Heird (1977). "Protection against experimental necrotizing enterocolitis by maternal milk. I. Role of milk leukocytes." Pediatr Res **11**(8): 906-9.

Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig and D. R. Marshak (1999). "Multilineage potential of adult human mesenchymal stem cells." Science **284**(5411): 143-7.

Ponti, D., A. Costa, N. Zaffaroni, G. Pratesi, G. Petrangolini, D. Coradini, S. Pilotti, M. A. Pierotti and M. G. Daidone (2005). "Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties." Cancer Res **65**(13): 5506-11.

Potten, C. S. and M. Loeffler (1990). "Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt." Development **110**(4): 1001-20.

Preffer, F. I., D. Dombkowski, M. Sykes, D. Scadden and Y. G. Yang (2002). "Lineage-negative side-population (SP) cells with restricted hematopoietic capacity circulate in normal human adult blood: immunophenotypic and functional characterization." Stem Cells **20**(5): 417-27.

Prentice, A. (1996). "Bioactive components of human milk." Proc Nutr Soc **20**: 146-155.

Prentice, A. M. and R. G. Whitehead (1987). The energetics of human reproduction. Reproductive Energetics in Mammals. A. S. I. Loudon and T. A. Racey. Oxford, Oxford University Press**:** pp 275-304.

Qi, H., D. J. Aguiar, S. M. Williams, A. La Pean, W. Pan and C. M. Verfaillie (2003). "Identification of genes responsible for osteoblast differentiation from human mesodermal progenitor cells." Proc Natl Acad Sci U S A **100**(6): 3305-10.

Reya, T., S. J. Morrison, M. F. Clarke and I. L. Weissman (2001). "Stem cells, cancer, and cancer stem cells." Nature **414**(6859): 105-11.

Richert, M. M., K. L. Schwertfeger, J. W. Ryder and S. M. Anderson (2000). "An atlas of mouse mammary gland development." J Mammary Gland Biol Neoplasia **5**(2): 227-41.

Richie, E. R., J. K. Hilliard, R. Gilmore and D. J. Gillespie (1987). "Human milk-derived lactoferrin inhibits mitogen and alloantigen induced human lymphocyte proliferation." J Reprod Immunol **12**(2): 137-48.

Risch, H. A., N. S. Weiss, J. L. Lyon, J. R. Daling and J. M. Liff (1983). "Events of reproductive life and the incidence of epithelial ovarian cancer." Am J Epidemiol **117**(2): 128-39.

Rodeheffer, M. S., K. Birsoy and J. M. Friedman (2008). "Identification of white adipocyte progenitor cells in vivo." Cell **135**(2): 240-9.

Rodriguez-Palmero, M., B. Koletzko, C. Kunz and R. Jensen (1999). "Nutritional and biochemical properties of human milk: II. Lipids, micronutrients, and bioactive factors." Clin Perinatol **26**(2): 335-59.

Rogan, W. J. and B. C. Gladen (1993). "Breast-feeding and cognitive development." Early Hum Dev **31**(3): 181-93.

Rohde, E., C. Malischnik, D. Thaler, T. Maierhofer, W. Linkesch, G. Lanzer, C. Guelly and D. Strunk (2006). "Blood monocytes mimic endothelial progenitor cells." Stem Cells **24**(2): 357-67.

Rosenblatt, K. A. and D. B. Thomas (1993). "Lactation and the risk of epithelial ovarian cancer. The WHO Collaborative Study of Neoplasia and Steroid Contraceptives." Int J Epidemiol **22**(2): 192-7.

Rousset, F., E. Garcia, T. Defrance, C. Peronne, N. Vezzio, D. H. Hsu, R. Kastelein, K. W. Moore and J. Banchereau (1992). "Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes." Proc Natl Acad Sci U S A **89**(5): 1890-3.

Rudland, P. S. (1987). Stem cells in mammary development and cancer. Cellular and Molecular Biology of Experimental Mammary Cancer. D. Medina, W. Kidwell, G. Heppner and E. Anderson. New York, Plenum Press**:** pp 9-28.

Rudland, P. S. (1991). "Generation of lobuloalveolar development from isolated rat mammary ducts and end buds." J Histochem Cytochem **39**(9): 1257-66.

Rudland, P. S., R. Barraclough, D. G. Fernig and J. A. Smith (1997). Mammary stem cells in normal development and cancer. Stem Cells. C. S. Potten. San Diego, Academic Press**:** pp 147-233.

Russo, J., Y. F. Hu, I. D. Silva and I. H. Russo (2001). "Cancer risk related to mammary gland structure and development." Microsc Res Tech **52**(2): 204-23.

Russo, J., R. Moral, G. A. Balogh, D. Mailo and I. H. Russo (2005). "The protective role of pregnancy in breast cancer." Breast Cancer Res **7**(3): 131-42.

Russo, J. and I. H. Russo (1987). Development of the human mammary gland. The Mammary Gland: Development, Regulation and Function. M. C. Neville and C. W. Daniel. New York, Plenum Press**:** pp 67-93.

Saarinen, U. M. and M. Kajosaari (1995). "Breastfeeding as prophylaxis against atopic disease: prospective follow-up study until 17 years old." Lancet **346**(8982): 1065-9.

Sakakura, T., Y. Nishizuka and C. J. Dawe (1976). "Mesenchyme-dependent morphogenesis and epithelium-specific cytodifferentiation in mouse mammary gland." Science **194**(4272): 1439-41.

Scariati, P. D., L. M. Grummer-Strawn and S. B. Fein (1997). "A longitudinal analysis of infant morbidity and the extent of breastfeeding in the United States." Pediatrics **99**(6): E5.

Schwertmann, A., S. Rudloff and C. Kunz (1996). "Potential ligands for cell adhesion molecules in human milk." Ann Nutr Metab **40**(5): 252-62.

Seale, P. and M. A. Rudnicki (2000). "A new look at the origin, function, and "stem-cell" status of muscle satellite cells." Dev Biol **218**(2): 115-24.

Sell, S. (1978). "Distribution of alpha-fetoprotein- and albumin-containing cells in the livers of Fischer rats fed four cycles of N-2-fluorenylacetamide." Cancer Res **38**(9): 3107-13.

Shackleton, M., F. Vaillant, K. J. Simpson, J. Stingl, G. K. Smyth, M. L. Asselin-Labat, L. Wu, G. J. Lindeman and J. E. Visvader (2006). "Generation of a functional mammary gland from a single stem cell." Nature **439**(7072): 84-8.

Shaheen, M. and H. E. Broxmeyer (2005). The humoral regulation of hematopoiesis. Hematology: Basic Principles and Practice. R. Hoffman, S. Benz, S. S.et al. New York, Churchill Livingstone**:** pp 233-265.

Sheibak, M. P., L. V. Evets and A. I. Pal'tseva (1978). "[Di- and monosaccharide makeup of breast milk and of basic substituent mixtures]." Vopr Pitan(4): 82-3.

Shirota, K., L. LeDuy, S. Y. Yuan and S. Jothy (1990). "Interleukin-6 and its receptor are expressed in human intestinal epithelial cells." Virchows Arch B Cell Pathol Incl Mol Pathol **58**(4): 303-8.

Shizuru, J. A., R. S. Negrin and I. L. Weissman (2005). "Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system." Annu Rev Med **56**: 509-38.

Shmelkov, S. V., R. St Clair, D. Lyden and S. Rafii (2005). "AC133/CD133/Prominin-1." Int J Biochem Cell Biol **37**(4): 715-9.

Shoham, Z. (1994). "Epidemiology, etiology, and fertility drugs in ovarian epithelial carcinoma: where are we today?" Fertil Steril **62**(3): 433-48.

Siimes, M. A., E. Vuori and P. Kuitunen (1979). "Breast milk iron--a declining concentration during the course of lactation." Acta Paediatr Scand **68**(1): 29-31.

Skansen-Saphir, U., A. Lindfors and U. Andersson (1993). "Cytokine production in mononuclear cells of human milk studied at the single-cell level." Pediatr Res **34**(2): 213-6.

Slade, H. B. and S. A. Schwartz (1987). "Mucosal immunity: the immunology of breast milk." J Allergy Clin Immunol **80**(3 Pt 1): 348-58.

Slayton, W. B. and G. J. Spangrude (2004). Adult stem cell plasticity. Adult Stem Cells. K. Turksen. Totowa, Humana Press**:** pp 1-18.

Sleeman, K. E., H. Kendrick, A. Ashworth, C. M. Isacke and M. J. Smalley (2006). "CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells." Breast Cancer Res **8**(1): R7.

Smaglik, P. (2001). "Fears of cults and kooks push Congress towards cloning ban." Nature **410**(6829): 617.

Smalley, M. and A. Ashworth (2003). "Stem cells and breast cancer: A field in transit." Nat Rev Cancer **3**(11): 832-44.

Smith, C. W. and A. S. Goldman (1968). "The cells of human colostrum. I. In vitro studies of morphology and functions." Pediatr Res **2**(2): 103-9.

Smith, G. H. (1996). "Experimental mammary epithelial morphogenesis in an in vivo model: evidence for distinct cellular progenitors of the ductal and lobular phenotype." Breast Cancer Res Treat **39**(1): 21-31.

Smith, G. H. (2002). "Mammary cancer and epithelial stem cells: a problem or a solution?" Breast Cancer Res **4**(2): 47-50.

Smith, G. H. (2006). "Mammary stem cells come of age, prospectively." Trends Mol Med **12**(7): 287-9.

Smith, G. H. and D. Medina (1988). "A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland." J Cell Sci **90 (Pt 1)**: 173-83.

Smith, G. H., P. Strickland and C. W. Daniel (2002). "Putative epithelial stem cell loss corresponds with mammary growth senescence." Cell Tissue Res **310**(3): 313-20.

Smith, J. A., R. Barraclough, D. G. Fernig and P. S. Rudland (1989). "Identification of alpha transforming growth factor as a possible local trophic agent for the mammary gland." J Cell Physiol **141**(2): 362-70.

Smith, J. A., J. Ham, D. P. Winslow, M. J. O'Hare and P. S. Rudland (1984). "The use of high-performance liquid chromatography in the isolation and characterisation of mouse and rat epidermal growth factors and examination of apparent heterogeneity." J Chromatogr **305**(2): 295-308.

Sommer, A., I. Tarwotjo, E. Djunaedi, K. P. West, Jr., A. A. Loeden, R. Tilden and L. Mele (1986). "Impact of vitamin A supplementation on childhood mortality. A randomised controlled community trial." Lancet **1**(8491): 1169-73.

Steinwender, G., G. Schimpl, B. Sixl, S. Kerbler, M. Ratschek, S. Kilzer, M. E. Hollwarth and H. H. Wenzl (1996). "Effect of early nutritional deprivation and diet on translocation of bacteria from the gastrointestinal tract in the newborn rat." Pediatr Res **39**(3): 415-20.

Sternlicht, M. D. (2006). "Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis." Breast Cancer Res **8**(1): 201.

Sternlicht, M. D. and Z. Werb (2001). "How matrix metalloproteinases regulate cell behavior." Annu Rev Cell Dev Biol **17**: 463-516.

Steward, C. G. and A. Jarisch (2005). "Haemopoietic stem cell transplantation for genetic disorders." Arch Dis Child **90**(12): 1259-63.

Stingl, J., C. J. Eaves, U. Kuusk and J. T. Emerman (1998). "Phenotypic and functional characterization in vitro of a multipotent epithelial cell present in the normal adult human breast." Differentiation **63**(4): 201-13.

Stingl, J., C. J. Eaves, I. Zandieh and J. T. Emerman (2001). "Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue." Breast Cancer Res Treat **67**(2): 93-109.

Stingl, J., P. Eirew, I. Ricketson, M. Shackleton, F. Vaillant, D. Choi, H. I. Li and C. J. Eaves (2006a). "Purification and unique properties of mammary epithelial stem cells." Nature **439**(7079): 993-7.

Stingl, J., A. Raouf, P. Eirew and C. J. Eaves (2006b). "Deciphering the mammary epithelial cell hierarchy." Cell Cycle **5**(14): 1519-22.

Storms, R. W., M. A. Goodell, A. Fisher, R. C. Mulligan and C. Smith (2000). "Hoechst dye efflux reveals a novel CD7(+)CD34(-) lymphoid progenitor in human umbilical cord blood." Blood **96**(6): 2125-33.

Strange, R., F. Li, S. Saurer, A. Burkhardt and R. R. Friis (1992). "Apoptotic cell death and tissue remodelling during mouse mammary gland involution." Development **115**(1): 49-58.

Streuli, C. H. (1993). "Extracellular matrix and gene expression in mammary epithelium." Semin Cell Biol **4**(3): 203-12.

Stuart, J., S. Norrell and J. P. Harrington (1984). "Kinetic effect of human lactoferrin on the growth of Escherichia coli 0111." Int J Biochem **16**(10): 1043-7.

Suzuki, H., T. Taguchi, H. Tanaka, H. Kataoka, Z. Li, K. Muramatsu, T. Gondo and S. Kawai (2004). "Neurospheres induced from bone marrow stromal cells are multipotent for differentiation into neuron, astrocyte, and oligodendrocyte phenotypes." Biochem Biophys Res Commun **322**(3): 918-22.

Szilvassy, S. J., R. K. Humphries, P. M. Lansdorp, A. C. Eaves and C. J. Eaves (1990). "Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy." Proc Natl Acad Sci U S A **87**(22): 8736-40.

Tabbara, I. A., K. Zimmerman, C. Morgan and Z. Nahleh (2002). "Allogeneic hematopoietic stem cell transplantation: complications and results." Arch Intern Med **162**(14): 1558-66.

Takala, A. K., J. Eskola, J. Palmgren, P. R. Ronnberg, E. Kela, P. Rekola and P. H. Makela (1989). "Risk factors of invasive Haemophilus influenzae type b disease among children in Finland." J Pediatr **115**(5 Pt 1): 694-701.

Takizawa, H., T. Ohtoshi, N. Yamashita, T. Oka and K. Ito (1996). "Interleukin 6-receptor expression on human bronchial epithelial cells: regulation by IL-1 and IL-6." Am J Physiol **270**(3 Pt 1): L346-52.

Tan, X. W., H. Liao, L. Sun, M. Okabe, Z. C. Xiao and G. S. Dawe (2005). "Fetal microchimerism in the maternal mouse brain: a novel population of fetal progenitor or stem cells able to cross the blood-brain barrier?" Stem Cells **23**(10): 1443-52.

Tappel, A. L. (1965). "Free-Radical Lipid Peroxidation Damage and Its Inhibition by Vitamin E and Selenium." Fed Proc **24**: 73-8.

Taylor-Papadimitriou, J. and E. B. Lane (1987). Keratin expression in the mammary gland. The Mammary Gland. M. C. Neville and C. W. Daniel. New York, Plenum Press**:** pp 181-215.

Taylor-Papadimitriou, J., E. B. Lane and S. E. Chang (1983). Cell lineages and interactions in neoplastic expression in the human breast. Understanding Breast Cancer. M. A. Rich, J. C. Hager and P. Furmanski. New York, Marcel Dekker**:** pp 215-246.

Taylor-Papadimitriou, J., R. Millis, J. Burchell, R. Nash, L. Pang and J. Gilbert (1986). "Patterns of reaction of monoclonal antibodies HMFG-1 and -2 with benign breast tissues and breast carcinomas." J Exp Pathol **2**(4): 247-60.

Taylor-Papadimitriou, J., M. Stampfer, J. Bartek, A. Lewis, M. Boshell, E. B. Lane and I. M. Leigh (1989). "Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to in vivo phenotypes and influence of medium." J Cell Sci **94 (Pt 3)**: 403-13.

Taylor-Papadimitriou, J., R. Wetzels and F. Ramaekers (1992). "Intermediate filament protein expression in normal and malignant human mammary epithelial cells." Cancer Treat Res **61**: 355-78.

Telemo, E. and L. A. Hanson (1996). "Antibodies in milk." J Mammary Gland Biol Neoplasia **1**(3): 243-9.

Temboury, M. C., A. Otero, I. Polanco and E. Arribas (1994). "Influence of breast-feeding on the infant's intellectual development." J Pediatr Gastroenterol Nutr **18**(1): 32-6.

Thompson, B. J. and S. Smith (1985). "Biosynthesis of fatty acids by lactating human breast epithelial cells: an evaluation of the contribution to the overall composition of human milk fat." Pediatr Res **19**(1): 139-43.

Thompson, P. A., F. F. Kadlubar, S. M. Vena, H. L. Hill, G. H. McClure, L. P. McDaniel and C. B. Ambrosone (1998). "Exfoliated ductal epithelial cells in

human breast milk: a source of target tissue DNA for molecular epidemiologic studies of breast cancer." Cancer Epidemiol Biomarkers Prev **7**(1): 37-42.

Thorgeirsson, S. S. (1996). "Hepatic stem cells in liver regeneration." Faseb J **10**(11): 1249-56.

Tiwari, A., G. Punshon, A. Kidane, G. Hamilton and A. M. Seifalian (2003). "Magnetic beads (Dynabead) toxicity to endothelial cells at high bead concentration: implication for tissue engineering of vascular prosthesis." Cell Biol Toxicol **19**(5): 265-72.

Toma, J. G., M. Akhavan, K. J. Fernandes, F. Barnabe-Heider, A. Sadikot, D. R. Kaplan and F. D. Miller (2001). "Isolation of multipotent adult stem cells from the dermis of mammalian skin." Nat Cell Biol **3**(9): 778-84.

Topper, Y. J. and C. S. Freeman (1980). "Multiple hormone interactions in the developmental biology of the mammary gland." Physiol Rev **60**(4): 1049-106.

Tropepe, V., B. L. Coles, B. J. Chiasson, D. J. Horsford, A. J. Elia, R. R. McInnes and D. van der Kooy (2000). "Retinal stem cells in the adult mammalian eye." Science **287**(5460): 2032-6.

Uauy, R., G. Stringel, R. Thomas and R. Quan (1990). "Effect of dietary nucleosides on growth and maturation of the developing gut in the rat." J Pediatr Gastroenterol Nutr **10**(4): 497-503.

Uchida, N., D. W. Buck, D. He, M. J. Reitsma, M. Masek, T. V. Phan, A. S. Tsukamoto, F. H. Gage and I. L. Weissman (2000). "Direct isolation of human central nervous system stem cells." Proc Natl Acad Sci U S A **97**(26): 14720-5.

Uchida, N., T. Fujisaki, A. C. Eaves and C. J. Eaves (2001). "Transplantable hematopoietic stem cells in human fetal liver have a CD34(+) side population (SP)phenotype." J Clin Invest **108**(7): 1071-7.

van 't Veer, L. J., H. Dai, M. J. van de Vijver, Y. D. He, A. A. Hart, M. Mao, H. L. Peterse, K. van der Kooy, M. J. Marton, A. T. Witteveen, G. J. Schreiber, R. M. Kerkhoven, C. Roberts, P. S. Linsley, R. Bernards and S. H. Friend (2002). "Gene expression profiling predicts clinical outcome of breast cancer." Nature **415**(6871): 530-6.

Van Buren, C. T., A. D. Kulkarni, W. C. Fanslow and F. B. Rudolph (1985). "Dietary nucleotides, a requirement for helper/inducer T lymphocytes." Transplantation **40**(6): 694-7.

Van Buren, C. T., A. D. Kulkarni, V. B. Schandle and F. B. Rudolph (1983). "The influence of dietary nucleotides on cell-mediated immunity." Transplantation **36**(3): 350-2.

Vassiliou, G., P. Amrolia and I. A. Roberts (2001). "Allogeneic transplantation for haemoglobinopathies." Best Pract Res Clin Haematol **14**(4): 807-22.

Visser, J. W., S. J. Bol and G. van den Engh (1981). "Characterization and enrichment of murine hemopoietic stem cells by fluorescence activated cell sorting." Exp Hematol **9**(6): 644-55.

Wagner, K. U., C. A. Boulanger, M. D. Henry, M. Sgagias, L. Hennighausen and G. H. Smith (2002). "An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal." Development **129**(6): 1377-86.

Waterworth, A. (2004). "Introducing the concept of breast cancer stem cells." Breast Cancer Res **6**(1): 53-4.

Watt, F. M. (1998). "Epidermal stem cells: markers, patterning and the control of stem cell fate." Philos Trans R Soc Lond B Biol Sci **353**(1370): 831-7.

Watt, S. M., S. E. Gschmeissner and P. A. Bates (1995). "PECAM-1: its expression and function as a cell adhesion molecule on hemopoietic and endothelial cells." Leuk Lymphoma **17**(3-4): 229-44.

Weiler, I. J., W. Hickler and R. Sprenger (1983). "Demonstration that milk cells invade the suckling neonatal mouse." Am J Reprod Immunol **4**(2): 95-8.

Wellings, S. R., H. M. Jensen and R. G. Marcum (1975). "An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions." J Natl Cancer Inst **55**(2): 231-73.

Welm, B., F. Behbod, M. A. Goodell and J. M. Rosen (2003). "Isolation and characterization of functional mammary gland stem cells." Cell Prolif **36 Suppl 1**: 17-32.

Welm, B. E., S. B. Tepera, T. Venezia, T. A. Graubert, J. M. Rosen and M. A. Goodell (2002). "Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population." Dev Biol **245**(1): 42-56.

Wiese, C., A. Rolletschek, G. Kania, P. Blyszczuk, K. V. Tarasov, Y. Tarasova, R. P. Wersto, K. R. Boheler and A. M. Wobus (2004). "Nestin expression--a property of multi-lineage progenitor cells?" Cell Mol Life Sci **61**(19-20): 2510-22.

Williams, J. M. and C. W. Daniel (1983). "Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis." Dev Biol **97**(2): 274-90.

Wilson, A. C., J. S. Forsyth, S. A. Greene, L. Irvine, C. Hau and P. W. Howie (1998). "Relation of infant diet to childhood health: seven year follow up of cohort of children in Dundee infant feeding study." Bmj **316**(7124): 21-5.

Wirt, D. P., L. T. Adkins, K. H. Palkowetz, F. C. Schmalstieg and A. S. Goldman (1992). "Activated and memory T lymphocytes in human milk." Cytometry **13**(3): 282-90.

Wiseman, B. S., M. D. Sternlicht, L. R. Lund, C. M. Alexander, J. Mott, M. J. Bissell, P. Soloway, S. Itohara and Z. Werb (2003). "Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis." J Cell Biol **162**(6): 1123-33.

Wold, A. E., C. Motas, C. Svanborg and J. Mestecky (1994). "Lectin receptors on IgA isotypes." Scand J Immunol **39**(2): 195-201.

Wolf, N. S., A. Kone, G. V. Priestley and S. H. Bartelmez (1993). "In vivo and in vitro characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection." Exp Hematol **21**(5): 614-22.

Woodward, W. A., M. S. Chen, F. Behbod and J. M. Rosen (2005). "On mammary stem cells." J Cell Sci **118**(Pt 16): 3585-94.

World Health Organisation (1989). “Protecting, promoting and supporting breastfeeding: The special role of maternity services.” Geneva, WHO.

Wright, A. L., C. J. Holberg, L. M. Taussig and F. D. Martinez (1995). "Relationship of infant feeding to recurrent wheezing at age 6 years." Arch Pediatr Adolesc Med **149**(7): 758-63.

Wright, M. H., A. M. Calcagno, C. D. Salcido, M. D. Carlson, S. V. Ambudkar and L. Varticovski (2008). "Brca1 breast tumors contain distinct CD44+/CD24- and CD133+ cells with cancer stem cell characteristics." Breast Cancer Res **10**(1): R10.

Wyllie, A. H., J. F. Kerr and A. R. Currie (1980). "Cell death: the significance of apoptosis." Int Rev Cytol **68**: 251-306.

Xanthou, M. (1997). "Human milk cells." Acta Paediatr **86**(12): 1288-90.

Xanthou, M., J. Bines and W. A. Walker (1995). "Human milk and intestinal host defense in newborns: an update." Adv Pediatr **42**: 171-208.

Yang, C. P., N. S. Weiss, P. R. Band, R. P. Gallagher, E. White and J. R. Daling (1993). "History of lactation and breast cancer risk." Am J Epidemiol **138**(12): 1050-6.

Yao, C. L., Y. H. Feng, X. Z. Lin, I. M. Chu, T. B. Hsieh and S. M. Hwang (2006). "Characterization of serum-free ex vivo-expanded hematopoietic stem cells derived from human umbilical cord blood CD133(+) cells." Stem Cells Dev **15**(1): 70-8.

Yin, A. H., S. Miraglia, E. D. Zanjani, G. Almeida-Porada, M. Ogawa, A. G. Leary, J. Olweus, J. Kearney and D. W. Buck (1997). "AC133, a novel marker for human hematopoietic stem and progenitor cells." Blood **90**(12): 5002-12.

Yoo, K. Y., K. Tajima, T. Kuroishi, K. Hirose, M. Yoshida, S. Miura and H. Murai (1992). "Independent protective effect of lactation against breast cancer: a case-control study in Japan." Am J Epidemiol **135**(7): 726-33.

Young, L. J., D. Medina, K. B. DeOme and C. W. Daniel (1971). "The influence of host and tissue age on life span and growth rate of serially transplanted mouse mammary gland." Exp Gerontol **6**(1): 49-56.

Yuan, J. M., M. C. Yu, R. K. Ross, Y. T. Gao and B. E. Henderson (1988). "Risk factors for breast cancer in Chinese women in Shanghai." Cancer Res **48**(7): 1949-53.

Zhang, Y., E. McNeill, H. Tian, S. Soker, K. E. Andersson, J. J. Yoo and A. Atala (2008). "Urine derived cells are a potential source for urological tissue reconstruction." J Urol **180**(5): 2226-33.

Ziegler, B. L., M. Valtieri, G. A. Porada, R. De Maria, R. Muller, B. Masella, M. Gabbianelli, I. Casella, E. Pelosi, T. Bock, E. D. Zanjani and C. Peschle (1999). "KDR receptor: a key marker defining hematopoietic stem cells." Science **285**(5433): 1553-8.

Zulewski, H., E. J. Abraham, M. J. Gerlach, P. B. Daniel, W. Moritz, B. Muller, M. Vallejo, M. K. Thomas and J. F. Habener (2001). "Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes." Diabetes **50**(3): 521-33.

APPENDICES

International Journal Publications

1. **Y Fan**, Rozen S, MSK Chong, ESM Lee, C Mattar, MA Choolani and JKY Chan "Identification and Characterization of Fetal Stem Cells in Various Regions of Fetal Brain." (Manuscript in Progress)

2. **Fan Y**, Chong YS, Choolani MA, Cregan MD and Chan JKY (2010). "Unravelling the Mystery of Stem/ Progenitor Cells in Human Breastmilk" PLoS ONE 5(12):e14421

3. MD Cregan, **Y Fan**, A Applebee, ML Brown, B Klopcic, J Koppen, LR Mitoulas, KME Piper, MA Choolani, YS Chong and PE Hartmann (2007). "Nestin-positive Cells in Human Breast milk." Cell Tissue Res. **329**(1): 129-36.

Posters/ Presentations

1. **Fan YP**, Mattar C, Choolani M, Chan J.Isolation and Characterization of Neural Stem Cells from Various Anatomical Regions of the Second Trimester Central Nervous System. The 7th International Congress of Obstetrics and Gynaecology, 26th-29th Aug 2009 (Abstract/ Poster Presentation)

2. **Fan YP**, Mattar C, Choolani M, Chan J. Identification of Neural Stem Cells In The Cerebral Cortex, Brain Stem, Spinal Cord And Hippocampus Of The Second Trimester Central Nervous System. International Society of Stem Cell Research 7th Annual Meeting, 8th-11th June 2009 (Abstract/ Poster Presentation)

3. **Fan YP**, Mattar C, Choolani M, Chan J. Identification of Neural Stem Cells In The Cerebral Cortex, Brain Stem, Spinal Cord And Hippocampus Of The Fetal Central Nervous System. 12th Annual Meeting of the American Society of Gene Therapy, 27th-30th May 2009 (Abstract/ Poster Presentation)

4. C Mattar, **Fan YP**, M Chong, M Choolani, YC Lim, J Chan. The Characterization of Fetal Neural Stem Cells (fNSC) from the Developing Fetal Brain: an In-vitro Model of Developmental Neurogenesis. "Pitch for Funds" Round of Clinician Scientist Unit (CSU).

5. **YP Fan**, J Chan, MD Cregan, MA Choolani, YS Chong. Do Stem/ Progenitor Cells Exist in Human Breast Milk? 7th Singapore Congress of Obstetrics and Gynaecology. 10th-13th Nov 2008 (Abstract/ Oral Presentation).

6. **YP Fan**, J Chan, J Ng, J Low, N Kothandaraman, A Ilancheran and M Choolani. Tumour-initiating Ability of Side-population of an Ovarian Cancer Cell Line.

NHG Annual Scientific Congress, Singapore, 7th-8th Nov 2008 (Abstract/ Poster Presentation).

7. **YP Fan**, J Chan, MD Cregan, MA Choolani and YS Chong. Do Stem/ Progenitor Cells Exist in Human Breast Milk? NHG Annual Scientific Congress, Singapore, 7th-8th Nov 2008 (Abstract/ Poster Presentation).

8. **Fan YP**, Chan J, Choolani MC, Chong YS and Cregan MD. Isolation of putative stem cells of multiple lineages from human breast milk. 14th International Conference of the International Society for Research in Human Milk and Lactation, 31st Jan-5th Feb 2008 (Abstract/ Poster Presentation)

9. **Fan YP**, Chan J, Chong YS, Cregan MD and Choolani MA. Identification and Characterization of Mammary Stem / Progenitor Cells in Human Breast Milk. International Society of Stem Cell Research 5th Annual Meeting, 17th-20th June 2007 (Abstract/ Poster Presentation)

10. Jerry Chan, **Fan Yiping**, Narasimhan Kothandaraman, Joseph Ng, Manuel Salto-Tellez, Mahesh Choolani. Isolation of Ovarian Cancer Stem Cells. "Pitch for Funds" Round of Clinician Scientist Unit (CSU).

11. **YP Fan**, MD Cregan, J Chan, MA Choolani, YS Chong. Presence of Stem/ Progenitor Cell Markers in Human Breast Milk. 6th Singapore Congress of Obstetrics and Gynaecology. 21st-25thMarch 2007 (Abstract/ Oral Presentation).

12. **Fan YP**, Chan J, Cregan MD, Choolani MA, Chong YS. Identification and Characterization of Mammary Stem/ Progenitor Cells in Human Breast Milk. NUS Obstetrics and Gynaecology Research Fair, 3rd – 4th March 2007 (Abstract/ Oral Presentation).

13. **YP Fan**, MD Cregan, J Chan, MC Choolani & YS Chong. Identification of a Side-Population (SP) of cells in Expressed Human Breast Milk: Novel Non-invasive Source of Undifferentiated Mammary Stem Cells. NHG Annual Scientific Congress, Singapore, 30th Sep – 1st Oct 2006 (Abstract/ Poster Presentation).

14. **Fan YP**, Chan J, Piper KME, Hartmann PE, Choolani MC, Chong YS & Cregan MD. Identification and characterization of mammary progenitor cells in human breast milk. 13th ISRHML International Conference, Canada, 22-26 Sept 2006 (Abstract/ Poster Presentation)

15. **YP Fan**, A Applebee, ML Brown, B Klopcic, J Koppen, LR Mitoulas, KME Piper, PE Hartmann, YS Chong & MD Cregan. Mammary Progenitor Cells in Expressed Human Breast Milk. Keystone Symposium: Stem Cells, Senescence and Cancer, Singapore, 25-30 October 2005 (Abstract/ Poster Presentation).

16. Y Qin, HM Zhang , N Kothandaraman, S Ponnusamy, **YP Fan**, N Primalani, WL Chua, YT Seet & M Choolani. Identification of Unique Membrane Proteins Associated with Red Blood Cells and Fetal Nucleated Red Blood Cells using

Tandem MALDI-TOF/TOF Approach. 8th NUS-NUH Annual Scientific Meeting, Singapore, 7-8 October 2004 (Abstract/ Poster Presentation).

17. N Mohammed, S Ponnusamy, M Rauff, AN Yong, KY Ngiam**, YP Fan**, A Biswas & M Choolani. Epsilon-Globin-Positive Primitive Fetal Erythroblasts in Trisomy 18 Neonates. 8th NUS-NUH Annual Scientific Meeting, Singapore, 7-8 October 2004 (Abstract/ Poster Presentation).

Awards

1. 6th Singapore Congress in Obstetrics and Gynaecology
   21st – 25th March 2007
   Oral Presentation
   Second Prize

2. NUS Obstetrics and Gynaecology Research Fair 2007, Singapore
   3rd – 4th March 2007
   Oral Presentation (Preliminary Studies Category)
   Second runner up