

Breast milk: a source of stem cells and protective cells for the infant

Microchimerism, the result of homing of foreign cells into our tissues, can occur during pregnancy and lactation. Although this natural transfer of viable cells including stem cells and immune protective cells from the mother to the infant and vice versa is likely to have a functional significance, this is yet unknown. New studies provide conclusive evidence of this phenomenon during the breastfeeding period. This review will summarise the current knowledge on breast milk cells and will present the potential significance and applications of these cells in the fields of lactation and medicine.

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Key points

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1. Breast milk contains nutritional components and also immunoprotective and developmental factors, including immune cells and stem cells.
2. Breast milk immune cells constitute a low proportion of cells in mature human milk, but they rapidly respond to infections of the mother or the infant.
3. Breast milk stem cells have multilineage potential and survive in the offspring, integrating and differentiating into functional cells in different neonatal tissues.
4. Breast milk cells can be used as a non-invasive and easy to access tool to assess the health status and functional performance of the lactating breast.

“A mother’s love is enduring”.¹ During fetal life and also in the first weeks to months after birth, the offspring depend entirely on the provision of nutrients by the mother via the placenta and breast milk, respectively.² In addition to nutritional material, the mother is also a source of cellular material to the fetus and the newborn infant. In fact, this occurs both ways during pregnancy, with a bi-directional exchange of cells, including stem cells. This is well documented to be facilitated via the placenta and to result in microchimeric offspring and mothers, whereby the fetus houses maternal stem cells and the mother receives embryonic stem cells.^{1,3}

Remarkably, the stem cells exchanged during pregnancy are thought to remain in the chimeras for life,¹ yet the evolutionary significance of this phenomenon is still unknown. Even less studied is the postpartum transfer of cells from the mother to the infant via breast milk. Indeed, groundbreaking studies have recently revealed that the maternal transfer of viable stem cells as well as immune protective cells to the offspring continues after birth during breastfeeding.^{4,7} What could be the fate of these cells?

In recent years, it has become obvious that breast milk is much more than nutrition, containing a plethora of bioactive molecules and maternal cells that are involved in various pathways of protection as well as infant development. Importantly, increasing evidence is demonstrating that these breast milk functions are species- and dyad-specific.^{4,8}

Breast milk is not sterile, being a rich source of bacteria that colonise the infant’s gastrointestinal (GI) tract and promote its normal development.⁹ Further to the prokaryotic cells, breast milk contains eukaryotic cells.⁴ These include both protective immune cells and a hierarchy of epithelial cells ranging from early-stage stem cells and their progeny, including progenitor cells and differentiated lactocytes and myoepithelial cells.⁴ Breast milk immune cells have been studied mostly in animal models, where they were shown to survive the infant gut and be transported to different tissues providing immunological support.^{10,11}

More recently, mouse studies have revealed the survival and transfer of a population of breast milk stem cells in the neonate’s GI tract, and passage to the systemic circulation from where they are transferred to different organs, integrating and differentiating into functional cells.⁷

Milk: a cellular fluid

It has been over 170 years since the first observation of cells in milk,^{12,13} yet it is only in the last decade that the rapid development of technologies for cellular and molecular analyses have enabled identification of numerous cell types with various functionalities in human milk. Although the initial research interest in cells from mammary secretions was targeted at accessing cells from the cancerous gland,^{14,15} more recently the focus has been on the normal lactating gland, its differences to the resting gland

(FIGURE 1) and the breast milk cell transfer to the breastfed infant.⁴

A great inter- and intra-individual variation in breast milk cellular content exists, ranging from ~10,000 to 13,500,000 cells/mL of milk.⁴ Numerous factors are known to influence this including:

1. the fullness of the breast, where emptier breasts contain milk with more cells than fuller breasts, similar to the changes in milk fat¹⁶
2. the stage of lactation; colostrum contains a higher number of cells than mature human milk¹⁷
3. the health status of the mother-infant dyad where, during infection of either the mother or the infant, breast milk contains significantly more immune cells, although the total cell content does not notably change¹⁷⁻¹⁹
4. the permeability of the basement membrane, and the development of the breast epithelium⁴
5. the methodological inconsistency between studies, which may account for a proportion of the variability in the literature, emphasising the need for standardisation of breast milk collection and optimisation of analytical assays.

What is consistent among studies and between women is the presence of two main cell categories in breast milk: epithelial cells and immune cells (leukocytes) (FIGURE 2).⁶ The relative proportions of these cells, their related cell subsets, and factors influencing them appear to be not only species-specific, but also characteristic of the mother-infant dyad.

Protective cells in human milk

During embryonic life, the mother provides the developing fetus with immune factors that protect the fetus from infection and contribute to the development of its intestinal mucosa, gut microflora and own defences.^{18,20,21} This process continues postnatally via breastfeeding, a crucial period that has been repeatedly shown to confer lower risk of disease in the long-term and significant reductions in infant and child mortality, both in developed and developing countries.^{22,23} In addition to bioactive molecules with immunocompetence such as immunoglobulins (secretory IgA, IgG, IgM), lactoferrin, lysozyme, oligosaccharides, cytokines and others, breast milk delivers daily thousands to billions of viable active cells (including immune cells) to the infant.^{4,17,18} This is

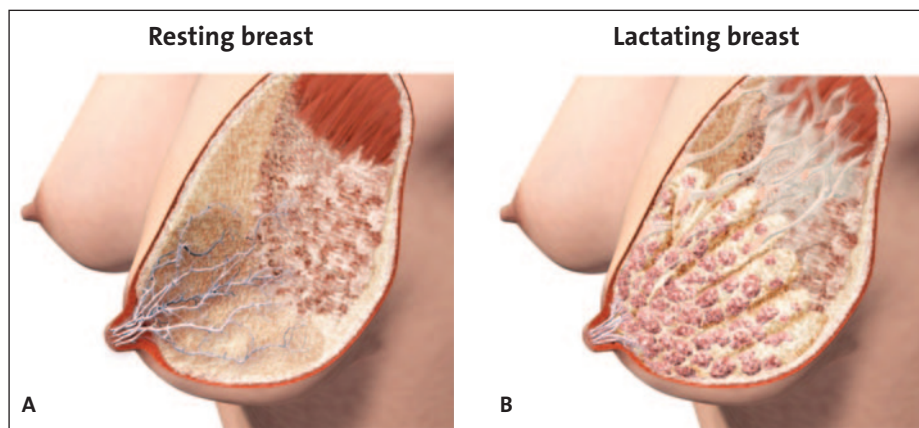


FIGURE 1 Anatomy of the human adult mammary gland (A) in its resting state (in non-pregnant, non-lactating women) and (B) its lactating state. Medela AG, 2012. Used with permission. Reproduced from Hassiotou and Hartmann.⁶

especially important as the infant's own immune system is immature at birth²⁴ and is thus susceptible to various infections. This is also consistent with the greater immune cell presence in colostrum in the first few days after birth, compared to mature human milk.^{4,17,18} At the same time, the mother passes onto her infant components of her innate immunity developed during her life, boosting the infant's start to life.⁴

Immune cells have been studied more extensively than any other cell type in breast milk, due mainly to their immunoprotective properties, but also due to the focus on bovine milk, which contains large quantities of immune cells.^{4,18} Milk immune cells include the main immune cell types typically found in blood: monocytes, macrophages, granulocytes, T and B lymphocytes.^{4,17,18,25-28} However, their migration and temporary homing inside the mammary gland is thought to result in programming of novel mammary-specific traits, which may be related to and aimed at their upcoming passage into the breast milk and the young.¹⁸ In both colostrum and mature human milk, the main immune cell type appears to be the monocyte/macrophage, with T and B lymphocytes constituting a low proportion (up to 10%), although this can change during periods of infection.^{4,18} Currently there is no evidence to suggest a difference in the types or proportions of immune cells between term and preterm human milk,²⁸ but this is an area that merits further investigation.

The majority of breast milk immune cells are activated, suggesting that they provide active immunity to the infant as illustrated by animal studies.^{4,17,18} Milk

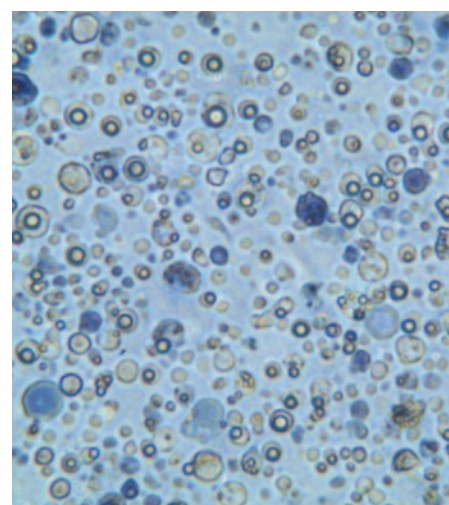


FIGURE 2 Cells isolated from freshly expressed breast milk and stained using trypan blue for cellular viability (photo by A.J. Twigger).

immune cells have been shown to be able to 'diapedese' or squeeze between mammary epithelial cells and enter the alveolar lumen.² Furthermore, they have been found in the bloodstream and organs of the young such as the mesenteric nodes, the liver and spleen, where they are thought to provide immunological support to the developing infant.^{10,11,29} More recent evidence in a mouse model has identified milk-derived immune cells in the thymus, liver, kidneys and spleen of the nursing young.⁷ These findings taken together provide strong evidence of survival of a proportion of milk immune cells in the GI tract of the breastfed offspring, and subsequent migration into the blood circulation and different organs, where they are likely to actively participate in the immunological maturation and immune defence of the offspring against pathogens.

Not only do milk immune cells have functions in the infant, but they also provide a reliable and species-specific tool that accurately reflects the health status of the mammary gland, and participates in its protection from pathogens during lactation.^{4,18} For example, during mastitis a significant increase in milk immune cell count has been reported both in women (up to 95% of total milk cells) and in the dairy cow. These immune cells are specifically recruited to the infected gland to fight infection, and this is accurately reflected into the milk expressed from the gland. Upon recovery, milk immune cell count returns to the low baseline level (<2.5% of total cells) that is representative of mature human milk, and thus of women's lactating breasts.¹⁷ In the dairy cow, somatic cell count also decreases after recovery from mastitis,³⁰ and is therefore a good test to assess the health status of the cow's udder.

In contrast, the somatic cell count (ie total cell count) is a poor representative of the health status of the lactating human breast, as evidence has shown that total cells do not necessarily increase during infection of the mother.¹⁷ Therefore, the measurement of immune cell count is the best test known to date that provides an assessment of the health status of the lactating breast in women.¹⁸ It is a simple and rapid tool that can detect a mammary, systemic or other local infection even before symptoms are manifested in either the mother or the infant.^{17-19,31} The author and colleagues have previously hypothesised that this breast maternal response to the infant's infection may be mediated by the mechanics of milk flow during breastfeeding,^{4,17} and it is an example of the multifunctional interaction between the mother and the infant that is facilitated by breastfeeding.

The epithelial cellular hierarchy and stem cells of human milk

Although in the early years of milk cell research, microscopically-based morphological assessments were primarily employed to identify and quantify the cells in breast milk, in more recent years these have been replaced by immunostaining methods that involve single cell analysis by flow cytometry. These methods are much more accurate and specific to the cell type of interest, and have resulted in a shift in the previously believed dogma that the dominant cell type in mature human milk

is the immune cell. In contrast to the milk of other mammalian species such as the cow, after the first ~1-2 weeks postpartum human milk is dominated by mammary epithelial cells that comprise >97.5% of total breast milk cells, except for periods of infection of either the mother or the infant.^{4,17,18,32}

Epithelial cells in human milk comprise ductal and alveolar cells, both of luminal and myoepithelial origin, that are either passively shed via exfoliation or actively migrate into breast milk.⁴ The majority of these cells are viable and can be isolated from breast milk and cultured. Findings from different studies have supported the existence of an epithelial cellular hierarchy in breast milk, including early-stage stem cells, progenitor cells that are in the process of differentiation, and more mature mammary epithelial cells such as lactocytes and myoepithelial cells.⁶ Breast milk stem cells possess unique properties, and they closely resemble human embryonic stem cells and other known types of pluripotent stem cells.⁵ They express pluripotency genes and can replicate and differentiate into multiple lineages. Breast milk stem cells typically create spherical structures *in vitro* that originate from a single breast milk stem cell and can expand clonally to create small organoids in the culture dish (FIGURE 3). These are visible with the naked eye and consist of primary, secondary and tertiary structures ending in alveoli-looking spheres, capable of synthesising and secreting milk components *in vitro*.^{4,6} Moreover, breast milk stem cells have been shown to differentiate into cells outside the mammary lineage, including neurons and glia, hepatocytes that synthesise albumin and other liver-specific factors, pancreatic beta-like cells that synthesise insulin, osteoblasts, chondrocytes, adipocytes and cardiomyocytes.^{4,6,33} Immunohistochemical analyses in both resting and lactating mammary tissues have demonstrated that breast milk stem cells originate at least in part from the mammary epithelium, where they are present in very low numbers in the resting gland,^{5,34} but are activated, potentially via hormonal stimuli, during pregnancy and lactation and replicate to facilitate the remodelling of the mammary gland into a milk-secretory organ.^{4,6}

These unique properties of breast milk stem cells are also present in embryonic stem cells, which are programmed to undergo multilineage differentiation in

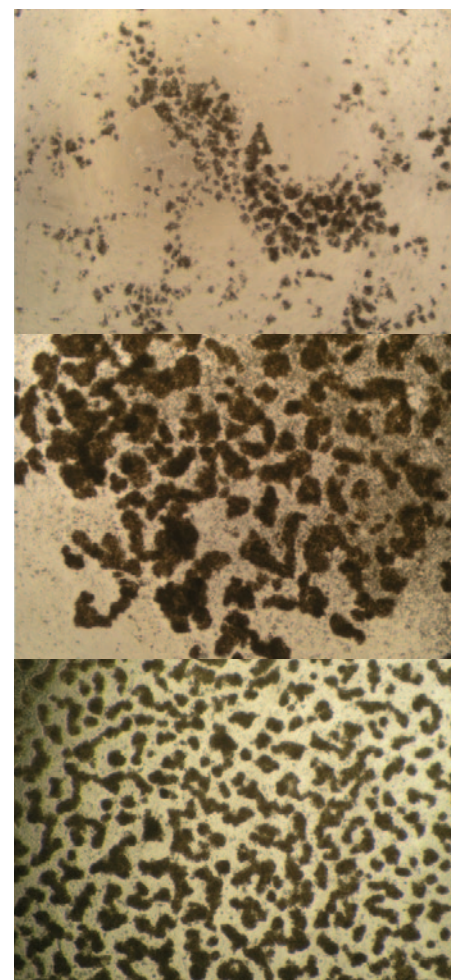


FIGURE 3 Spheroids and small organoids formed from the *in vitro* propagation of breast milk stem cells.

order to create the developing organism. Very little is known about somatic stem cells that postnatally display similar properties. In addition to breast milk, such cells have been detected in small numbers in the bone marrow and in some reproductive organs, however, their properties and role(s) are still poorly understood.^{4,5,35,36} All of these normal somatic stem cells are incapable of forming tumours in the teratoma assay, similar to breast milk stem cells. In contrast, embryonic stem cells and induced pluripotent stem cells respond positively in the teratoma assay.^{4,6,35,36} Although this property most likely results from postnatal epigenetic modifications aimed at preserving the cellular integrity of tissues and organs,^{4,6,36} it has inadvertently questioned the pluripotent nature of these somatic stem cells. Nevertheless, it has been suggested that the teratoma assay is not appropriate to detect pluripotency in normal non-tumorigenic cells, with the best alternative assay being the *in vivo*

cellular integration in normal tissues.⁴

With the aim of illustrating this, a mouse model has been employed recently to investigate the fate of breast milk stem cells in the neonate. Remarkably, although some cellular fragments were present in the stomach of the suckling pups, viable stem and immune cells derived from breast milk survived the GI tract of the young and were found in the bloodstream and in different organs such as the thymus, liver, pancreas, spleen, kidneys and brain.⁷ There, evidence of cell integration and tissue-specific differentiation was found, suggesting that breast milk stem cells participate in the development and function of the neonate's organs early in life.⁷ Furthermore, breast milk cells were found in the blood of the suckling pups after weaning, demonstrating that they are maintained through to adulthood. This study provided the first evidence of breast milk stem cell integration and multilineage differentiation *in vivo*, which further supported the previously discussed pluripotent nature of these cells. It has also paved the way for further investigations of the functional significance of these cells for the infant and of factors that may influence the numbers and/or properties of these cells in breast milk.

The cellular content of human milk is known to vary widely both between and among lactating women.^{4,5} A recent study examined maternal as well as infant characteristics that may explain this variation.³⁷ Interesting associations were found with maternal body mass index (BMI), the gestational age of the infant at birth, the stage of lactation, and infant sex. As it has been shown that the cells in mature human milk under healthy conditions directly represent the lactating epithelium,²⁴ any breast milk cell changes might be indicative of lactation status/performance. Indeed, it was found that women with high BMI (in the obese range) had significantly fewer lactocytes, providing a potential molecular explanation of and intervention trait for the management of the low milk supply that is typical of obese mothers.³⁷ These hypotheses must be confirmed in more directed studies in the future. Term milk was shown to contain more lactocytes than preterm milk, also leading to the speculation that the breasts of mothers with premature infants in some cases may have not reached the level of maturity required for copious milk synthesis.³⁷

Interestingly, gene expression differences were found between the milk of women with boys versus girls,³⁷ which are consistent with previous reports on animal milk supporting a fetus-driven differential remodelling of the mammary gland.³⁸ The study also reported on cellular changes the breast undergoes during the course of lactation and how they may relate to the reduced risk of breast cancer³⁷ as well as to the development of a lactation memory that facilitates the remodelling of the gland at the next pregnancy.^{37,39}

The future of breast milk cell research

The presence of viable multifunctional stem cells in human milk, which survive in the breastfed infant and naturally integrate into its tissues to potentially confer developmental signalling, has sparked enormous interest in the possibility of banking these stem cells for later therapeutic applications. Currently, this research is in its infancy. Perhaps the first step of using breast milk stem cells medically would be to increase their consumption in the fragile immature preterm infant. While most neonatal intensive care units (NICUs) recognise that mother's own milk is ideal for the infant, due to organisational constraints the milk is often fed to the infants after being frozen or pasteurised, in the case of donor milk. Since milk cells remain viable for a few hours after expression of the milk and they do not typically survive after freezing or pasteurisation, any potential benefits are largely withheld from preterm infants in the NICU. Animal studies have demonstrated necrotising enterocolitis (NEC)-protective functions of various types of stem cells provided to the neonate, with the greatest survival observed in the breastfed group,⁴⁰ suggesting that this protection could be mediated by milk stem cells and potentially other milk components. In addition to the preterm infant, breast milk stem cells may be used medically in infants with genetic diseases or other life-threatening conditions as a natural stem cell therapy. Indeed, there is a unique tolerance between the mother and her infant, which begins *in utero* and is enhanced postnatally during the breastfeeding period. This tolerance supports the potential use of breast milk cells as a natural therapy for neonates, but perhaps also for adults.⁴ Breast milk cells not only have potential benefits for the

infant and may be used medically, but they also provide a non-invasive and easily accessible tool to assess the health and performance of the mother's breasts. Assaying the milk immune cell subsets is an easy tool to monitor the progress of breast infections during treatment, and could be particularly useful in managing and treating mastitis to facilitate timely recovery and continuation of breastfeeding. At the same time, breast milk epithelial cells represent the lactating mammary epithelium, and thus its function, providing a tool to assess lactation performance and aiding the management of low milk supply.

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