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Original article

Human breast milk stem cells: a new challenge for perinatologists

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Stem cells: present and future
Guest Editors: Gavino Faa (Cagliari, Italy), Vassilios Fanos (Cagliari, Italy), Antonio Giordano (Philadelphia, USA)

Abstract

The lactating mammary gland contains a stem cells population with multilineage potentialities. Recently it was also shown that breast milk contains a heterogeneous population of stem cells that have the potential to differentiate *in vitro*, under the control of specific differentiation conditions, into mammary epithelial as well as into adipogenic, chondrogenic and osteogenic cell lineages. While the different types of cells present in the milk is known, what is less understood is the proportion of different milk cell types, their significance for the mother and the infant and factors influencing them.

In this manuscript we summarize some of the latest knowledge from *in vivo* and *in vitro* investigations on breast milk stem cells, we discuss their potential functions and applications and we present some of our preliminary data obtained in fresh human breast milk cells.

Keywords

Stem cells, human breast milk, milk stem cells markers, CD44, Ki67.

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Introduction

Recent researches concerning the isolation and characterization of stem cells, the discovery of signaling pathways involved in their self-renewal and survival, and their potential role in diseases (such as cancer) highlight the potential of these cells and we now start to discuss a potential use of adult stem cell plasticity in bioengineering and tissue regeneration as well as new strategies of cancer therapies [1].

Stem cells are distinguished from other cell types because they have the ability to self-renew and to develop into more differentiated cells. Therefore, when a stem cell divides, each new cell has the potential either to remain a stem cell or to become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, or a brain cell.

One of the most important element able to govern the stem cells capacity is the local environment, called the stem cells niche. Biological signal generated in the niche by cell adhesion or by interaction between different types of cells is thought to be one the control mechanism of stem cells activity [2, 3].

We may distinguish two kinds of stem cells: the most primitive embryonic stem cells and the adult stem cell. The embryonic stem cells have an extraordinary differentiation potential and can mature into a fully developed organism. The adult stem cells are generally quiescent cells that may generate new stem cells or more committed progeny or both [4, 5].

Stem cells buffered within specialized niches in adult tissues maintain homeostasis during tissue turnover or injury and represent a sort of internal repair system [6].

For example, the mammary gland is metabolically active, having the capacity to undergo cycles of extensive proliferation and hypertrophy (during pregnancy and lactation) and involution. The presence of mammary stem cells are necessary in order to give rise to different components of the lactation process. These cells proliferate extensively and differentiate during each pregnancy and lactation and undergo apoptosis during mammary involution [7, 8].

In order to identify and to study the functional properties of stem cells, several *in vitro* systems have been proposed. The task has been proved to be technically difficult because of the scarcity of stem cells in the tissue of origin, and the lack of universal morphologic traits for stem cells [9].

Recently it has been reported that breast milk contains a heterogeneous cell population; among this, a subpopulation with stem cell properties (including the ability to differentiate into different cell lineages) has been isolated from human fresh milk [10, 11]. Stem cells detected in mammary gland tissue with the differentiation capacity to originate mammary epithelial cell and myoepithelial cells [12] have been considered as the sources of stem cells in breast milk [13]. The presence of exfoliated epithelial cells from alveoli, macrophages, and lymphocytes was also reported [14, 15]. It was also observed that 10-15% of the cells isolated from fresh breast milk expressed mesenchymal stem cell (MSC) markers; culturing these isolated cells led to an increase in the MSC population, due to their higher capacity of cell proliferation [10].

Breast milk has long been known to contain maternal cells [16], but only recently it has been possible to distinguish, among these cells, immune cells and milk stem cells. For decades, milk was considered only from a nutritional point of view and just recently we have started to study a possible role of protective cells in breast milk. As the mammary gland is characterized by a significant remodeling during pregnancy, also the milk composition changes, not only in the nutritional elements, but also in the presence of different patterns of cells during lactation [17, 18].

While the different types of cells present in the milk is known, their significance and factors influencing them are less well understood. Only few data are available regarding the human stem cell composition in the fresh maternal human milk, but some information regarding the functions and characteristics of these cells comes from *in vitro* and animals experimental studies.

Recently, using female genetically modified mice whose cells contain a gene called *tdTomato*,

which makes them red under fluorescent light, it has been found that in unmodified lactating mice breastfed by *tdTomato*, female red stem cells cross into the offspring's blood through their stomach and play a functional role later in life. When the offspring reached adulthood, red cells were found in their blood and in many of their tissues, including brain, thymus, pancreas, liver, spleen and kidneys. These data suggest a possible migration and a functional integration into organs of maternal milk stem cells in the neonate [19].

Breast milk comprises epithelial cells, colostral corpuscles, polymorphonuclear leukocytes, mononuclear phagocytes, lactocytes and lymphocytes forming the main bulk of cells within two weeks of lactation [14, 20-22].

It was hypothesized that these epithelial cells are shed from the ductal and luminal epithelial layers through either a heightened turnover of the secretory tissue, or as a consequence of the mechanical shear forces associated with the continued filling and emptying cycle associated with breast milk synthesis and lactation [23].

Markers of breast milk stem cells

These putative mammary stem cells present in the milk were first identified through their expression of various cytokeratin (CK) markers (like CK5, CK14 and CK19) and nestin [24] but, even if in some cases there are contrasting data, other hallmarks of milk stem/progenitor cells have already been established [23].

By isolating and expanding MSC-like cells population from human breast milk, it is now possible to study their pluripotency using various culture conditions [25]. These cultured cells, examined by immunofluorescent labeling, were positive for MSC surface markers (like CD44, CD29, Sca-1) and negative for CD33, CD34, CD45, CD73, confirming their identity as MSCs. Cytoskeletal protein marker analysis revealed that these cells expressed MSCs markers, namely nestin, vimentin, smooth muscle actin. Moreover, it also manifested presence of E-cadherin, an epithelial-to-mesenchymal transition marker in their early passages. Also the multipotent differentiation potential of stem cells was proved; they can differentiate into adipogenic, chondrogenic and osteogenic lineage under the control of specific *in vitro* differentiation systems. This means that these MSCs, isolated from human breast milk, could potentially be "reprogrammed" to form many types of human tissues [26].

Breast milk as a new source of endogenous stem cells

The presence of multipotent stem cells in human milk suggests that breast milk could be an alternative source of stem cells for autologous stem cell therapy, although the actual significance of these cells needs to be more delineated.

It was also shown the presence of a cellular hierarchy in breast milk, from multipotent stem cells to progenitors to more differentiated mammary cells [11, 17].

The cell concentration in milk may range widely from 1×10^3 to 8×10^5 cells per mL, which seems to be not related to the duration of lactation [14, 23]. In another study, the total number of cells obtained from 1 mL of breast milk ranged between 2.5 and 3 million cells [26, 27].

Freshly isolated human breast milk cells contained 10-15% of CD44, CD29 and Sca-1 positive cells, and, when isolated and cultured *in vitro*, these cells significantly increased up to 90% after 4 in culture confluence passages. The immunofluorescence study for specific cell surface markers clearly indicated that these cells express mesenchymal markers (like SMA, vimentin, nestin) and surface markers (like CD44, CD29, Sca-1). These cells were found to be negative for CD33, CD34, CD45, CD73, confirming their identity as MSCs [27].

Large inter- and intra-individual variations exist in cell breast milk composition and it seems that they are correlated with breastfeeding stimulation, week of gestation and stage of lactation, but these variations are still not well understood. Probably this phenomenon is responsible for the variability of the published data; it is time now to establish a standardization of sampling protocols in lactation studies and investigation of the mechanisms of milk synthesis and cell movement into breast milk [28]. The majority of the published data regarding the human breast milk cells are obtained in cells culture experiments, using different *in vitro* conditions and probably, for this reason, these informations are in some cases in contrast and difficult to analyze. Therefore, as previously suggested, the standardization of sampling protocols for studies of breast milk cells is very important and still more data need to be determined in the fresh milk.

Preliminary personal data

In this contest, our preliminary results in fresh human breast milk cells suggest that one

of the best marker for the identification of MSCs in human milk is the surface marker CD44. In order to use standard conditions for immunohistochemical process, in these experiments fresh human milk was centrifuged and then the pellet was stored in commercial Cytological ThinPrep® solution (Hologic® Inc.). Microscope cytological slides were obtained using the automatic ThinPrep® processor (Hologic® Inc.) and these slides were used for standard immunohistochemical reactions performed with an automated stainer (Dako).

Fig. 1 shows that only few cells are CD44 positive, suggesting that this marker may discriminate, among the total cells present in the milk, some putative MSCs.

These are single cells or, in some cases, are grouped as two or more cells together, which may be CD44 positive, or both positive and negative. In another experiment, using different fresh human milk samples, we obtained the variation of the CD44-positive cells during different gestational and lactation periods (**Fig. 2**). These preliminary results suggest that, in our experimental conditions, the

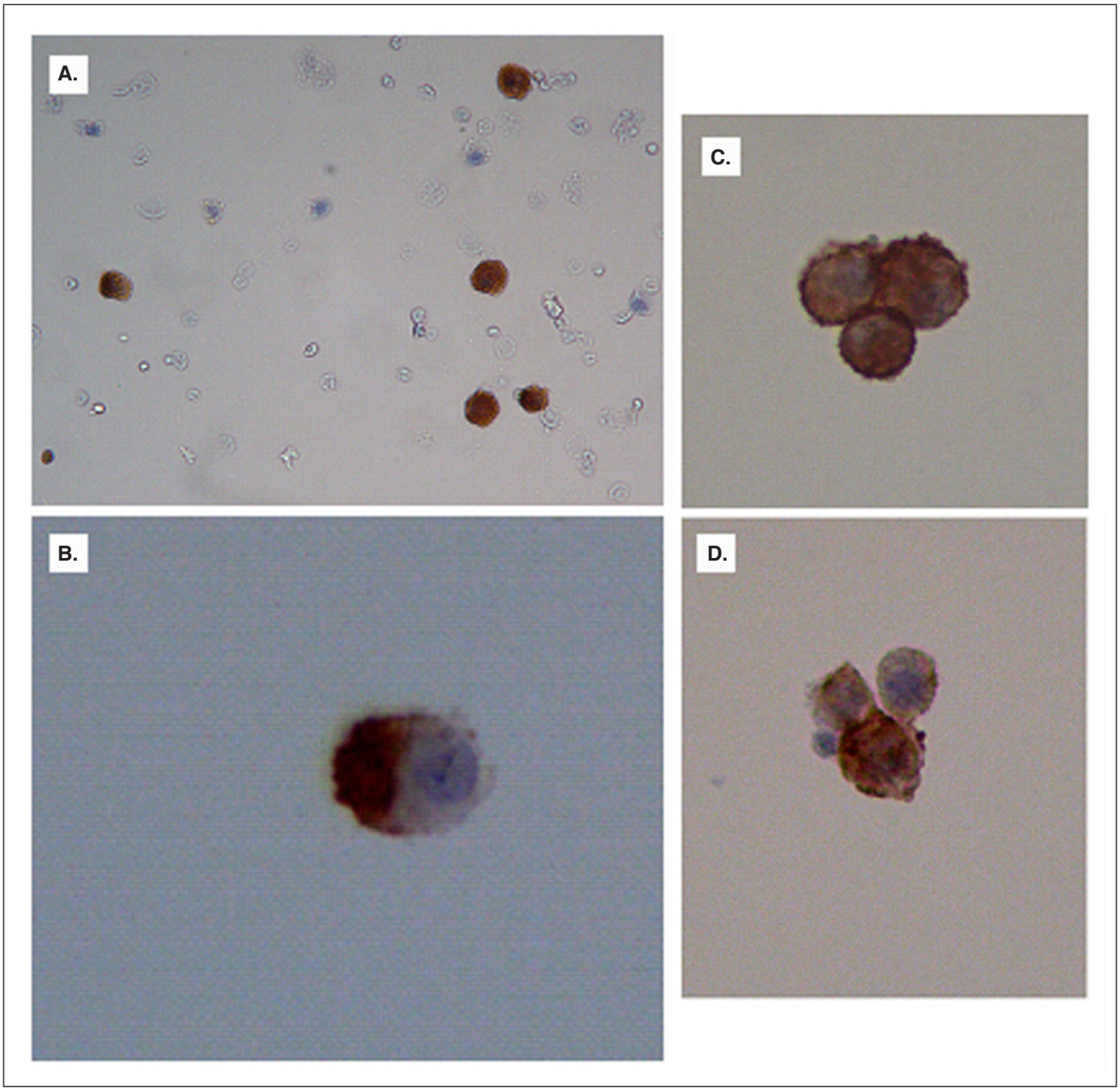

Figure 1. Putative mesenchimal stem cells of human breast milk positive for the CD44 immunohistochemical reaction. **A.** Total cells founded in fresh human breast milk, only few cells are CD44 positive. **B.** A single cell half positive for CD44. **C.** and **D.** A group of fresh human breast milk cells with different expression of CD44 marker.

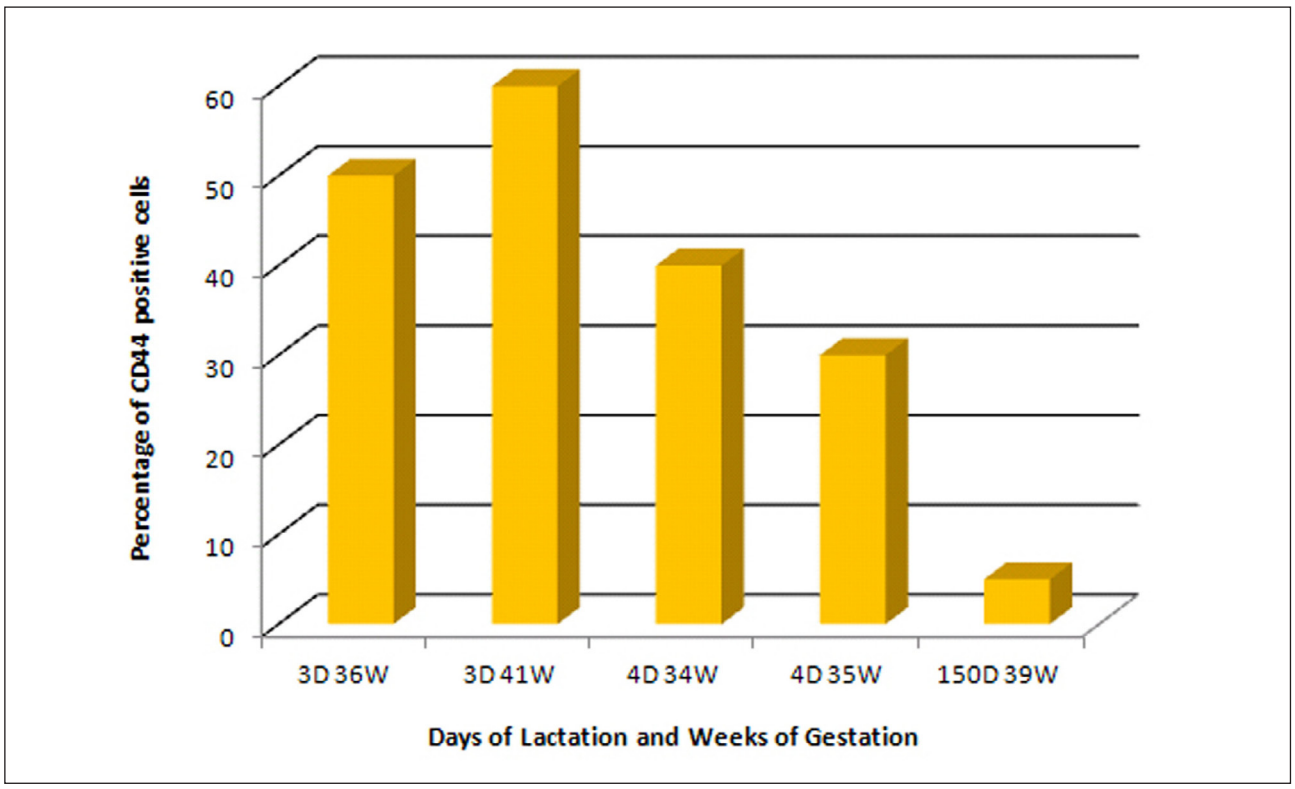

Figure 2. Graphic representation of the percentage of CD44-positive fresh human breast milk cells during different weeks of gestation (W) and days of lactation (D).

percentage of CD44-positive cells might decrease during lactation.

Although stem cells are believed to divide infinitely by self-renewal division, it is not clear if they have infinite replicative potential. Several reports suggest that also the mechanism of programmed cell death in this type of cells could be different as compared to that of normal somatic cells [29].

Evidence for extrinsic aging and various aging markers relating to morphology, proliferation, signaling, senescence markers, telomeres and telomerase, and other indicators start to be investigated also in stem cells [30].

We try to investigate if, in fresh human breast milk, a cell cycle marker like Ki67 (MIB-1) may give some information about the state of the cells present in this biological fluid.

As it is shown in **Fig. 3**, a few percentage of cells resulted positive for this marker and, among these, one was characterized by the presence of several fragment of the nucleus, all positive for the immunoreactions of the marker Ki67 (**Fig. 3C**). Nucleus fragmentation is a typical apoptotic event; therefore, this cell could be an hypothetical stem cell that, after proliferation, was blocked in a phase of the cell cycle and then was eliminated by apoptosis. This possibility is in part supported by some evidences previously published [29, 31].

Conclusions

We can summarize the importance of stem cells with the final prospective written by the 2007 Nobel Prize winner in Physiology and Medicine Martin J. Evans: "With the advent of human embryonic stem cells and the possibilities of using them as a renewable source of tissue-specific precursors for tissue transplant therapies and regenerative medicine, the importance of understanding and controlling embryonic stem cell determination and differentiation *in vitro* has been highlighted. It is clear that the utility of isolation, maintenance and use of pluripotential stem cells has a long and important future" [32].

Finally, coming back to breast milk stem cells, our preliminary data indicate these as a fascinating field of research in which, thanks to the cooperation of perinatologists, biologists and molecular pathologists, we might obtain new data on the complex relationship between the lactating mother and the newborn after birth.

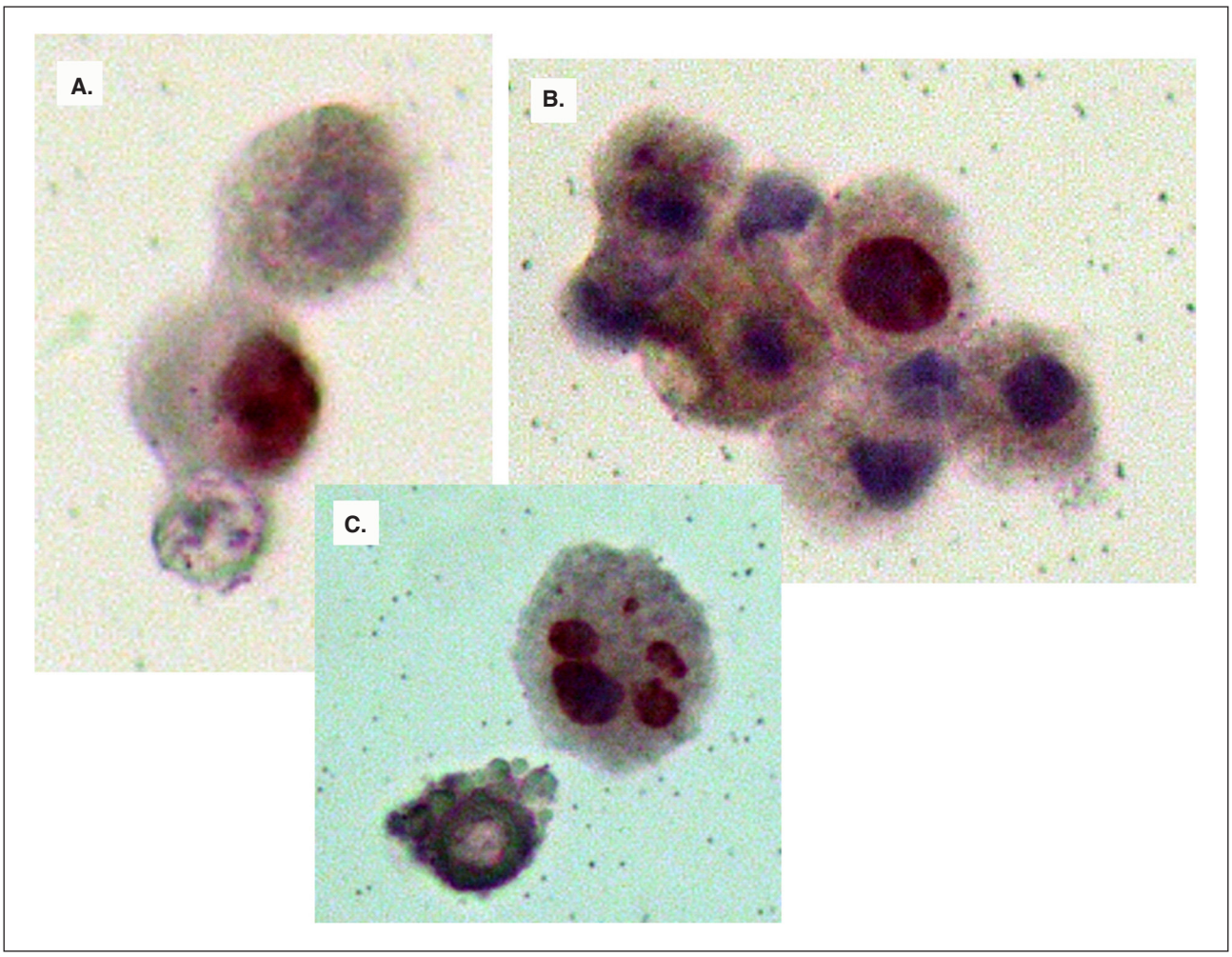

Figure 3. Fresh human breast milk cells positive for the Ki67 immunohistochemical reaction. **A.** and **B.** Two groups of cell with only one Ki67-positive cell. **C.** Apoptotic cell with several Ki67-positive nuclear fragments.

Declaration of interest

The Authors declare that there is no conflict of interest.

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