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Human breast milk cells are positive for the pioneer transcription factor ISL1

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Abstract. – OBJECTIVE: ISL1 is a pioneer transcription factor that plays important roles in cell lineage specification and differentiation, by programming the epigenome and recruiting additional regulatory factors. The aim of this study is to determine whether the human breastmilk contains ISL1-positive stem cells, and, if so, to describe the subcellular localization of ISL1.

MATERIALS AND METHODS: Breast milk was obtained from fourteen healthy females during the first 2-6 months of lactation. Cell morphology was examined in the breast milk with the automatic ThinPrep® processor (Hologic® Inc.) in commercial Cytological ThinPrep® solution (Hologic® Inc.), followed by standard immunohistochemical staining of ISL1.

RESULTS: ISL1 had a granular diffuse cytoplasmic localization, with varying intensity of staining in both single and grouped cells. Nuclear staining was also present, as was staining of intracellular and extracellular vesicles with ISL1 antibody.

CONCLUSIONS: These preliminary results suggest that ISL1 could distinguish a readily available source of putative stem cells in human breast milk. These stem cells may complete the network created between the mother and the newborn during gestation, thereby improving the efficiency of programming and reprogramming postnatal events.

Key Words:
ISL1, Immunohistochemistry, Stem cells, Breastmilk, Transcription factors.

Introduction

The mammary gland is a metabolically active dynamic organ that undergoes significant changes during pregnancy, lactation, and involution[1]. A recent work[2] has suggested that stem cells are an integral component of human breast milk, and they are identified through their expression of various stem cell markers. Certain types of cells present in breast milk are able to pass through the infant's gastrointestinal tract and populate distant sites such as the spleen, liver, and lymph nodes[3]. By isolating and expanding this adult stem cell population, it has been possible to study their pluripotency in various cell culture conditions and confirm their ability to differentiate into different cell lineages[4,5].

There is communication between breast milk components and the natural host, and some researchers consider this system a temporary organ[6-8]. The maintenance of stem/progenitor cells and their differentiation fate in mammary milk follows a well-defined genetic/epigenetic program. This program is implicated in controlling the homeostatic balance between self-renewal and differentiation state during pregnancy and lactation. Even though results may be strongly influenced by the *in vitro* conditions used[9], breast milk cultured stem cells have been shown to be reactive for multiple mesenchymal stem cell markers like *CD44*, *CD29*, *SCA-1*, and negative for others like *CD33*, *CD34*, *CD45*, *CD73*, confirming their identity as mesenchymal stem cells[10].

It has been proposed that, similar to other mesenchymal stem cell compartments, a hierarchical organization of cells exists within milk that has the potential to program or reprogram various types of human tissue, thereby contributing to proper lactation growth and development[8,11].

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An example of the capacity of stem cells to change in progressive subgroup type during embryogenesis and afterward was recently described during cardiomyocyte maturation. In this case, an important role is played by the ISL1 transcription factor, which is able to influence at least two or three different cell lineages of cardiomyocytes[12-14].

ISL1 is a *LIM-HD* transcription factor, originally identified as a protein that can bind to an insulin gene enhancer and regulate its expression[15]. The ISL1 gene contains six exons, located on chromosome 13 in mouse and chromosome 5 in human. The LIM-homeodomain (HD) proteins are an evolutionarily conserved superfamily of transcription factors that play fundamental roles in cell differentiation, cell fate determination, and the generation of cell diversity and segmental patterns during embryogenesis[16].

Cardiac ISL1 expression is transient and promotes successive conversion into an early cardiomyocyte identity. It seems that rather than stabilizing a transient precursor, ISL1 serves to accelerate specific cardiac cell differentiation[17]. Several studies[18-22] have also revealed that ISL1 is expressed in multiple tissues and cell types, including islet cells[18], neurons[19], cardiac progenitor cells (CPCs)[12], incisor epithelium[20], hindlimb bud cells[21], and in the developing human uterus[14,22].

ISL1 is expressed in multiple organs not only during embryogenesis but also during the entire lifespan[23].

Recent studies[24] have revealed multifaceted roles for ISL1 in epigenetic and transcriptional regulation. Interestingly, ISL1 may act as a pioneer transcription factor to shape the chromatin landscape. Pioneer factors are a specialized subset of transcription factors, with the capacity to bind specific DNA sequences within closed chromatin, triggering remodeling of the chromatin landscape to allow access of non-pioneer transcription factors[25]. In this study we analyze the possibility that mesenchymal milk stem cells could be positive for ISL1, suggesting a role for these cells in milk development.

Materials and Methods

Breast Milk Cells

Breast milk cells were isolated from 14 healthy voluntary females during the first 2-6 months of lactation. Fresh milk, diluted with an equal volume of Dulbecco's phosphate-buffered saline (DPBS; pH 7.4; Gibco; Fisher Scientific Italia, Strada Rivoltana Km 4, 20053 Rodano, MI, Italy) was centrifugated at 810 g for 20 min at 20°C. The fat layer and liquid were removed, and the cell pellet was washed twice with DPBS. The pellet was fixed with Thin-Prep solution (Hologic, Inc. www.hologic.com), and the cell samples were processed for immunocytochemistry analysis using cell slides prepared with the Thin Prep 5000 Processor (250 Campus Drive Marlborough, MA, USA). The presence of milk cells was monitored in slides colored with hematoxylin-eosin.

Immunocytochemistry Staining

Antibodies for ISL1 were used for immunocytochemistry. Sample preparation was performed using standard validated cytologic protocols (ThinPrep Preserv Cyt solution, ThinPrep 5000 system, https://www.hologic.com). The Ventana automated stains system (http://diagnostics.roche.com) was used for immunocytochemistry staining, and a qualitative analysis was performed by three different pathologists, comparing the reactivity in slides of the same sample using an individual scoring system (morphological parameters, % of number of stained cells, area, and intensity of the stain).

Results

Figure 1 shows an example of cells isolated with simple centrifugation from a sample of fresh human milk. These cells were fixed and stained using cytological methods routinely used in our

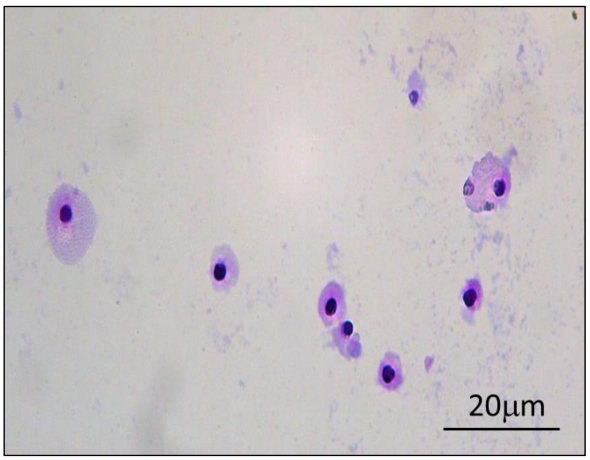

Figure 1. An example of cells isolated with a simple centrifugation from fresh human milk. These cells, fixed and stained using our cytological methods routinely used in our laboratories, are characterized by different morphological aspects, underling the presence of a heterogeneous cell population.

laboratories and have variable morphology, underling the presence of a heterogeneous cell population.

As for other stem cell markers already described in the literature, ISL1 distinguishes a subclone of staminal cells with different morphological characteristics (Figure 2). In particular, we found mono and binucleate cells (Figure 2 B, C) with different greatness (Figure 2A).

We generally observed granular diffuse cytoplasmatic expression in grouped and single positive cells (Figure 3). Additionally, we observed a range of intensity levels of ISL1 expression within both single and grouped cells (Figure 4 A, D, E), varying from a few spots to intense cytoplasmic positivity (Figure 4 B, D). Rare cases of nuclear positivity were also noted, as shown in Figure 5.

Another peculiarity of ISL1-positive cells is the presence of intracellular (Figure 6 A-B) or extracellular (Figure 6 C, D, E, F) vesicles. In some cases, these vesicles are ISL1-positive (Figure 6 A, B, C, D, E, F) and in other cases, they are negative for ISL1 (Figure 6 E, F).

It is worth noting that exosomes and vesicles have already been described in breast milk and that the presence of various molecules inside these vesicles could be an important strategy of cell communication[26].

Discussion

Mammals are, in part, defined by breastfeeding, but our understanding of the components of human milk is still limited. Stem cell and non-stem cell constituents of human milk have recently been intensely studied. Fascinating ideas have emerged from these reports, relating to the biological mechanisms of infant-mother communication[2,27,28].

Previous research[2,29] has demonstrated that human maternal milk contains a significant population of hematopoietic and mesenchymal stem cells, as well as a larger number of myoepithelial progenitors, cell adhesion molecules, immune cells, growth factors, and vesicles. This report represents the first description of a subpopulation of milk cells characterized by ISL1 positivity, adding to the growing understanding of the complex composition of human maternal milk.

The homeodomain transcription factor LIM ISL1 plays a key role in several cell-to-cell communication modes. ISL1 plays essential roles in cell proliferation, differentiation, and survival during both embryonic and postnatal life. The number and the type of ISL1-positive cells varies amongst diverse species including mice, rats, and humans[13].

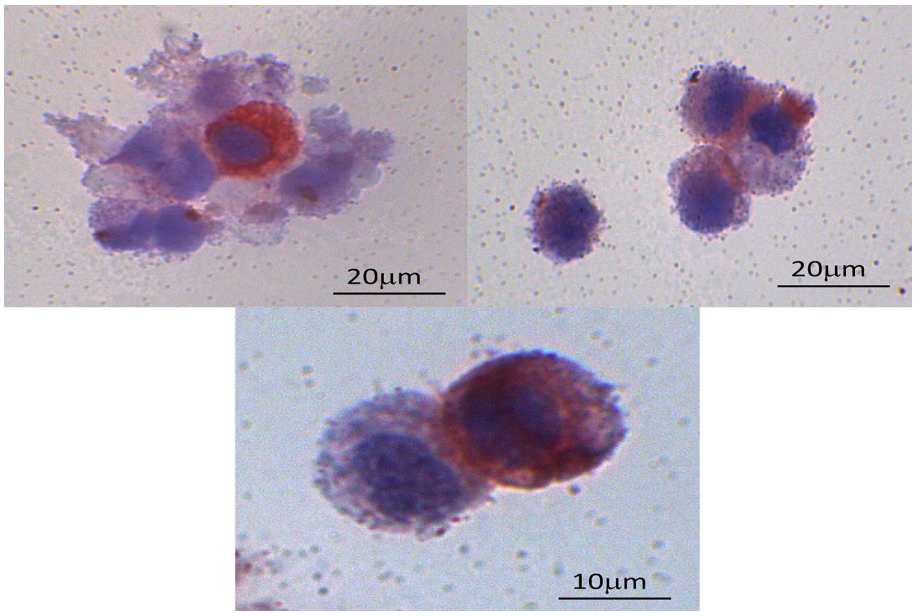

Figure 2. An example of cells isolated with a simple centrifugation from fresh human milk. These cells, fixed and stained using our cytological methods routinely used in our laboratories, are characterized by different morphological aspects, underling the presence of a heterogeneous cell population.

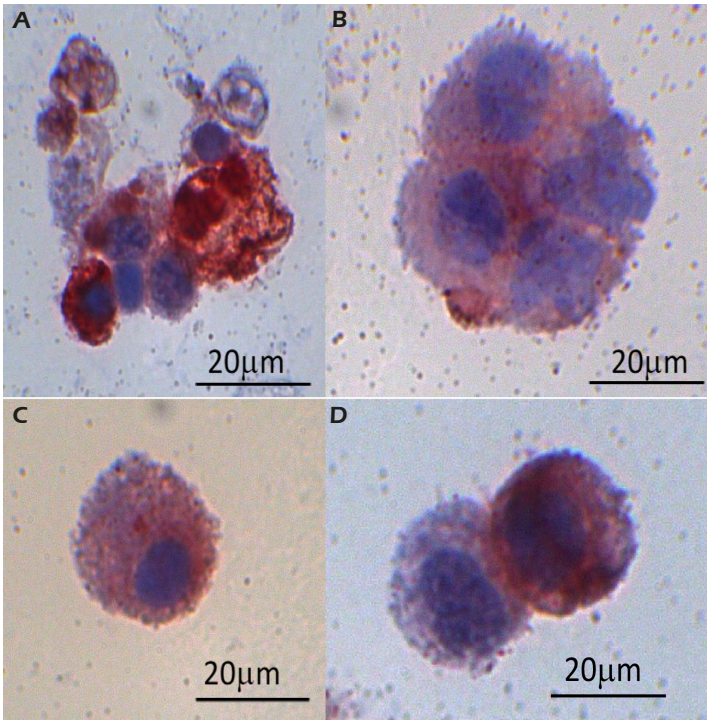

Figure 3. Grouped (**A, B**) and single (**C, D**) ISL1 positive cells, with a granular diffuse cytoplasmatic expression.

Even if ISL1 exerted a specific role in the development of the pancreas, the expression and functions of this protein have been extensively studied in different organs.

ISL1 positively modulates melatonin synthesis in the pineal gland and plays an important role in maintaining the daily circadian rhythm[30]. This protein was defined as an undifferentiated precursor in the embryonic and adult heart[31]. It is also involved in nervous system growth[32-34], forming two different types of multi-protein complexes in the spinal cord and forebrain and triggering the

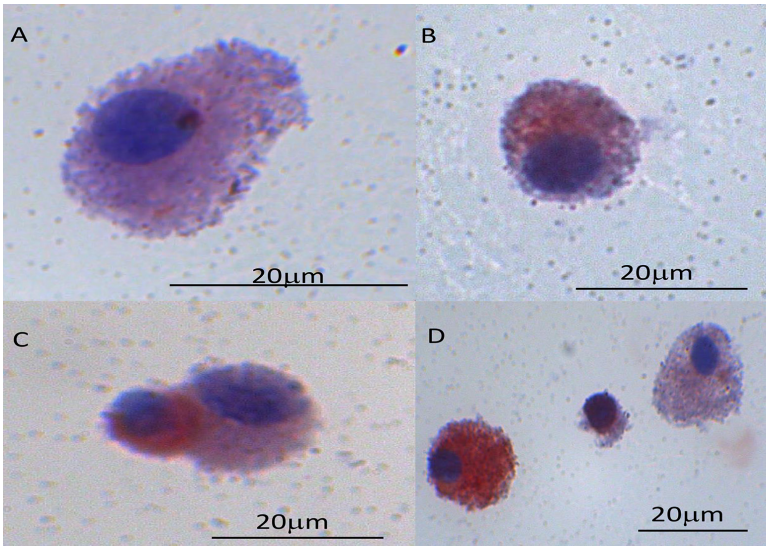

Figure 4. The intensity of the ISL1 expression is variable from a few spots (**A-B**) to an intense cytoplasmatic positivity (**C-D**).

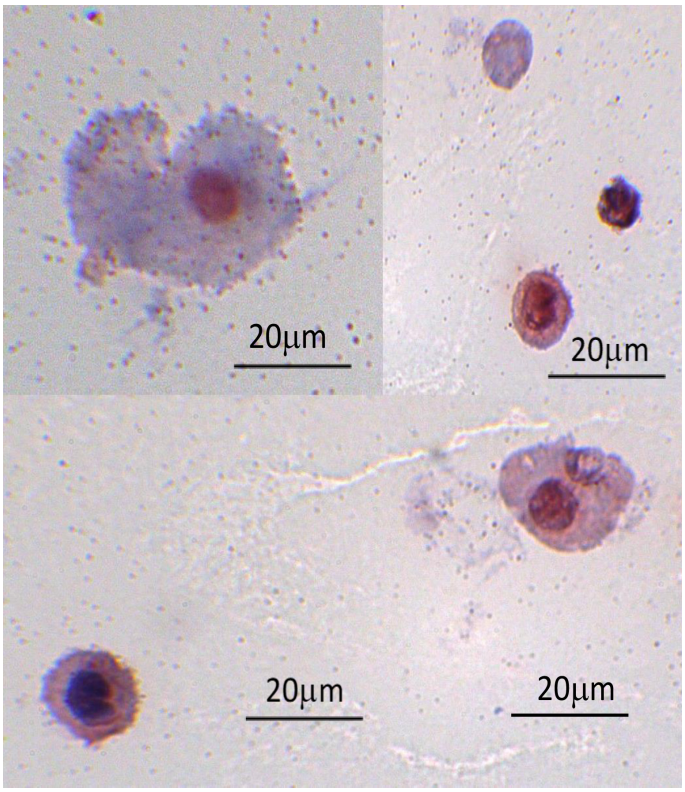

Figure 5. In rare cases, ISL1 nuclear positivity was also noted.

generation of cholinergic neurons in embryonic neural stem cells. Interestingly, these cholinergic genes include enzymes that synthesize acetylcholine and proteins required to package acetylcholine into vesicles[14,35], something which we also found in our samples (Figure 6).

One possible explanation for the presence of ISL1-positive cells in maternal milk is that the human brain rapidly develops during the first two years following birth[36]. The possible, pioneering transcription function of ISL1 in the epigenetics of cardiomyocyte cell fate[37-38] and post-natal

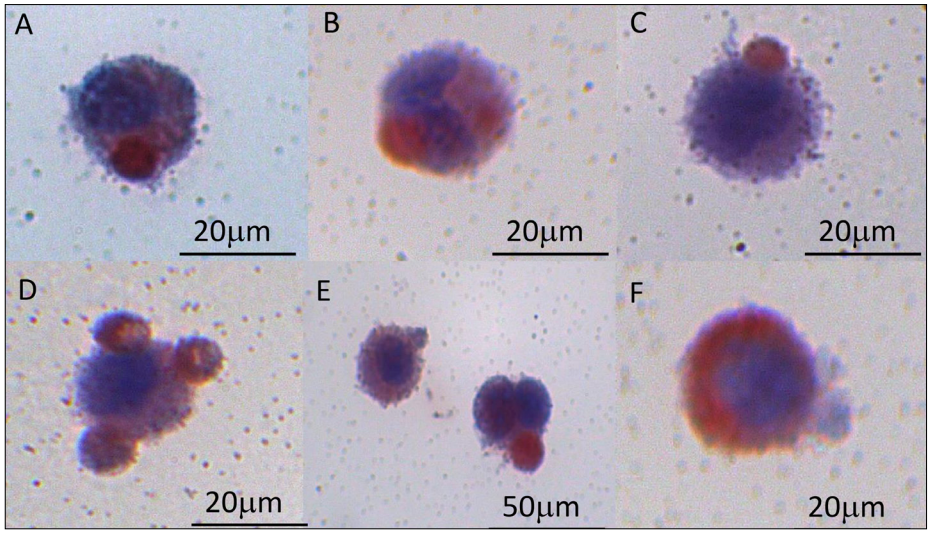

Figure 6. ISL1 positive and negative cells with intracellular (**A-B**) and extracellular vesicles (**C-D**). In some cases, these vesicles are ISL1 positive, and in others negative (**E-F**).

angiogenesis and vasculogenesis[39] were recently studied.

Pioneer transcription factors have the ability to scan partial DNA sequence motifs that are exposed on the surface of a nucleosome and thus access silent genes that are inaccessible to other transcription factors[40]. This unique ability to open closed chromatin and activate gene expression is important in developmental networks[41]. The presence of ISL1 in the nucleus, as shown in Figure 5, could be an indication of this pioneer transcription activity.

Inappropriate expression of pioneer transcription factors and the creation of chimeric pioneers through mutations or chromosomal translocations have also been described during neoplastic evolution[42].

It has been shown that ingested stem cells in milk can be transferred in the neonatal body to different compartments. It is, therefore, plausible that these ISL1-positive cells may contribute to the control of postnatal cellular embryonic precursor compartments.

From this perspective, the breastfeeding period can be considered a progression of the fetal prenatal programming postnatally. As a pioneer transcription factor of cell fate, ISL1 can engage naïve chromatin to induce hierarchical lineage-specific regulatory networks, improving the efficiency of programming and reprogramming postnatal event[13,43,44].

The presence of ISL1-positive cells in milk and their potential role in lactation development adds to the growing understanding of the complex interactions and communication between the mother and the neonate during this period.

Conclusions

In conclusion, this preliminary study suggests a possible role for milk staminal cells and *ISL1*-positive vesicles in neonatal development. The possibility that the growth of some organs may continue after birth underlines the role of breastfeeding as a continuous and beneficial activity during postnatal growth. Further studies are required to understand the precise nature of *ISL1*-positive breast milk stem/progenitor cells and to explore their potential clinical applications, as recently suggested[45].

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ethics Approval

All procedures were approved by the Ethic Human Studies Committee of the University Medical Centre of Cagliari (No. Prot. PG/2022/795) and were conducted according to the instructions of the Declaration of Helsinki.

Informed Consent

All participants provided written informed consent.

Availability of Data and Materials

Data is contained within the article. Dr. Pichiri G had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final version before submission.

Authors' Contribution

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