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Original article

Cellular distribution of some intermediate filaments in the rat mammary gland during pregnancy, lactation and involution

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Abstract

Intermediate filaments (IFs) play a major role in determining and maintaining cell shape and anchoring intracellular organelles in place, in the tissues and organs of several species, starting from the early stages of development. This study was aimed at the immunohistochemical investigation of the presence, cellular localization and temporal distribution of the intermediate filaments keratin 8 (CK8), keratin 18 (CK18), keratin 19 (CK19), vimentin, desmin and laminin, all of which contribute to the formation of the cytoskeleton in the rat mammary gland during pregnancy, lactation and involution. On days 7, 14 and 21 of pregnancy (pregnancy period), on day 7 post-delivery (lactation period) and on day 7 post-weaning (involution period), under ketamine hydrochloride (Ketalar-Pfizer) (90 mg/kg) anesthesia, two mammary glands were fully excised from the abdominal region. It was determined that CK8 showed moderate immunoreactions in the alveolar and ductal epithelia, connective tissue and vascular endothelium of the rat mammary gland throughout pregnancy. On the 7th day of pregnancy, CK18 expression was absent in the alveolar and ductal epithelia, but was observed weakly in some connective tissue cells. Throughout pregnancy, lactation and involution, the alveolar and ductal epithelia of the rat mammary gland were determined to be negative for CK19. Desmin expression predominated in the mammary myoepithelium and vasculature throughout all three of the investigated periods. While vimentin was not expressed in any of the mammary tissue components during pregnancy and lactation, its moderate expression was observed in the alveolar and ductal epithelia during involution. The involution period was also characterized by the vimentin negativity of the myoepithelium, stroma, fat cells and blood vessels of the mammary gland. Throughout all three periods, laminin expression was strong in the alveolar and ductal epithelia, stromal and myoepithelial cells and blood vessels, and did not vary in strength between the investigated periods. These findings demonstrated that intermediate filaments showed cell- and tissue-specific expression patterns in the rat mammary gland under the effects of pregnancy, lactation and involution.

Keywords: cytokeratin, desmin, involution, mammary gland

Introduction

The mammary gland is a complex tubuloalveolar gland embedded in irregular connective tissue. Milk is produced in the distally located differentiated acini of the gland and is transported to the proximally situated nipple in a series of branching duct glycoproteins (Russo and Russo 2004). The intralobular ducts, which are the smallest of the mammary ducts, together with the alveolar clusters form the lobules, otherwise known as the terminal ductal lobular units (TDLUs). The mammary parenchyma is mainly composed of two types of differentiated epithelial cells. Luminal cells are cuboidal/columnar cells, which line both the ducts and the alveoli. During lactation, the luminal cells of the distal ducts and alveoli start to produce milk, and thereafter are referred to as alveolar cells. Myoepithelial cells are muscular epithelial cells, which are situated between the luminal cells and basal membrane and have contractile property. In the ducts, the myoepithelial cells form a sheath. These cells are more sparsely distributed in the alveoli, and their cytoplasmic structure allows some of the alveolar epithelial cells to contact the basal membrane and form a basket-like structure (Emerman and Vogl 1986).

The cytoskeleton of mammalian tissue and organ cells is built of three main filament networks, namely, the microfilaments, microtubules and intermediate filaments (IFs). Keratins, neurofilaments, vimentin and desmin are the largest subgroup of IFs found in cells. The size of the IFs ranges between that of the microtubules, which measure approximately 25 nm in diameter, and that of microfilaments, which measure approximately 7 nm in diameter. IFs make up part of the insoluble cytoplasmic filaments of cells. IFs have been detected in almost all differentiated eukaryotic cells. By means of immunological, biochemical and molecular techniques, IFs have been detected in various cells and tissues, including cytokeratins in epithelial cells, desmin in muscle cells, neurofilaments in neurons, and vimentin in mesenchymal cells (Steinert et al. 1984).

Among IF proteins, cytokeratins (CKs) are mostly localized in epithelial cells. The majority show organ- or tissue-specific expression. The particular cytokeratin expressed by an epithelial cell depends on the type, terminal differentiation stage, and developmental phase of the cell. Thus, specific cytokeratin expressions enable the identification of epithelial cells (Sun et al. 2010). There are two types of cytokeratins, namely, acidic type I cytokeratins (CK9-CK20) and basic or neutral type II cytokeratins (CK1-CK8). Some cytokeratins serve as lineage markers of mammary epithelial cells. For instance, while CK8 and CK18 are found in the luminal epithelial cells of the normal mammary gland, CK5 and CK14 are localized to the basal or myoepithelial cells. On the other hand, in addition to luminal epithelial cells, CK7 and CK19 are sometimes expressed in basal cells (Sun et al. 2010). CK19 is described as being a neutral cytokeratin and its expression in the human mammary gland serves as a luminal marker (Bartek et al. 1990). Furthermore, CK14/CK19-positive transitional cells have been detected in the adult human mammary gland and have been claimed to be multipotent progenitor cells (Villadsen et al. 2007). However, there is no literature report available on whether CK14/CK19-positive cells are also found in the mammary glands of mice and rats. There is a paucity of information on the details of the expression of these cytokeratins in the mammary gland during embryonic and postnatal development, pregnancy, lactation and involution. In fact, increased information would enable a better understanding of how specific cell differentiation occurs during mammary gland development. Moreover, more detailed data would aid in identifying the embryonic origin of adult mammary stem/progenitor cells and contribute not only to our basic understanding of epithelial cell biology, but also to research on mammary gland cancers.

Vimentin, a 57 kDa-protein, is the most commonly expressed member of the family of type III IF proteins. Normally, it is expressed by mesenchymal cells (Raymond and Leong 1989), but its presence has also been determined in the mammary glands of humans and other mammals (Hellmén and Lindgren 1989). Furthermore, there are reports on the detection of vimentin expression in pancreatic progenitor cells, Sertoli cells, neurons, trophoblastic giant cells, fibroblasts, and endothelial, renal tubular and stromal cells, as well as macrophages, neutrophils and leukocytes (Madekurozwa 2013).

Desmin is the characteristic IF protein of myogenic cells and is encoded by a single gene (Rangdaeng and Truong 1991). It is the primary IF protein expressed by the cardiac, skeletal and smooth muscles. It interacts with other proteins to form a continuous cytoskeletal network, which establishes a spatial relationship between the contractile apparatus and other structural components of the cell, and thereby enables the maintenance of cellular integrity, force transmission, and mechanochemical signals. In particular, desmin is found at much higher levels in the myocardium, compared to skeletal muscle, and is described as one of the main components of the Purkinje fibres, which form a specialized transmission system that ensures the regular contraction of the myocardium (Goldfarb and Dalakas 2009). Desmin has been reported to be expressed also by fibrous tissue during wound healing and in some cells of the tumoral stroma. Moreover, it has been

shown to be expressed in the human and dog mammary glands together with some IFs (Hellmén and Lindgren 1989). In contrast, the normal mammary gland of the cat has been reported to be devoid of desmin expression (De Las Mulas et al. 2009).

Laminin is a major component of the basal membrane and contains glycoproteins (Streuli et al. 1995). Although 18 laminin isoforms have been described to date, the in vitro presence of some of them still needs to be confirmed (Timpl and Brown 1994). Laminins are heterotrimers, which show temporally regulated tissue-specific expression and are composed of the α-, β- and γ-subunits (Ahmed and Ffrench-Constant 2016). Various laminin isoforms have been detected in the mammary gland, and previous studies have shown that the most common isoforms in the adult mammary gland are laminin-111 (containing the α1, β1 and γ1 subunits), laminin-332, laminin-511/521, laminin-211 and laminin-411/421 (Gudjonsson et al. 2002). Some other studies have reported Lama1 and Lama as the most common laminins in the basal cells of the mammary gland, and Lama5 as the most common in the luminal cells of the mammary gland (Lim et al. 2010, Bach et al. 2017). However, the exact role of the spatial and temporal expression of laminins in the maintenance of the development and functioning of the normal mammary gland remains unclear.

The mammary gland is a complex tissue, which has evolved to feed the newborn and has several physiological, biochemical and immunological functions (Anand et al. 2012). Over the life span of female mammals, the mammary gland undergoes a series of changes during pregnancy, lactation and involution. Throughout the reproductive cycle, multiple consecutive regenerative processes, characterized by cell proliferation and terminal differentiation, occur in the epithelial layer of the mammary gland. It is considered that the expansion of the epithelial component of the mammary gland during pregnancy is initiated by the mitotic division of mammary stem cells and results in the formation of a mammary pool of progenitor cells. The progenitor cell population enters a highly proliferative state, which drives this epithelial expansion and is followed by cell differentiation. During post-lactational involution, the regenerative cycle ends with extensive apoptosis and tissue reformation (Li et al. 2007). These changes are regulated by the complex interaction of various hormones and molecular mechanisms. This cellular and functional complexity draws attention to the investigation of the role of various components in the functioning of the mammary gland. Today, it is necessary to investigate the presence and fully understand the physiological role of the molecular factors localized into the mammary gland as it produces milk, which has an important place in nutrition. In this context, the present study was designed with the aim of determining the cellular and periodic localization of several IFs, including CK8, CK18, CK19, vimentin and desmin, and a connective tissue component, laminin, all which contribute to the structure of the cytoskeleton, in the rat mammary gland during pregnancy, lactation and involution. It was considered that the clarification of the cell-, organ- and period-specific expression patterns of IF proteins in the rat mammary gland could provide novel information on the potential roles of these proteins, which depend on hormonal interaction.

Materials and Methods

Ethical statement for the experimental animals and experimental study conditions

Thirty-five adult female Sprague-Dawley rats, which weighed 220-250 g and were supplied from the Prof. Dr. Sabahattin PAYZIN Health Sciences Research and Application Center of Dicle University, were used in this study. The animals were housed under a 12-h light and 12-h dark cycle and were provided ad libitum access to pellet feed and drinking water. The rats were randomly assigned to 5 groups, each of 7 animals. All female rats were mated with two male rats. Pregnancy examinations were performed using the vaginal smear method. Animal, for which the presence of spermatozoa and the development of a vaginal plaque were determined in the vaginal smear samples, were considered as being in the first day of their pregnancy (Sağsöz and Ketani 2010). This study was approved by the Local Ethics Board for Animal Experiments of Dicle University (DÜHADEK) (Decision Number: 2008-02). The rats used in the study were euthanized without harming them within the framework of ethical rules. It was approved by the Dicle University Experimental Animals Local Ethics Committee (DÜHADEK) (Decision number 2008-2).

Collection and processing of the tissue samples

On days 7, 14 and 21 of pregnancy (pregnancy period), on day 7 post-delivery (lactation period) and on day 7 post-weaning (involution period), under ketamine hydrochloride (Ketalar-Pfizer) (90 mg/kg) anesthesia, two mammary glands were fully excised from the abdominal region. The experimental animals received postoperative care. In each group, mammary tissue samples were taken and fixed in 10% neutral formalin solution for 24 h. The samples were then washed under running water for another 24 h. The tissue samples were

then passed through a graded series of alcohol, methyl benzoate and benzol, and embedded in paraplast. Five-micrometer-thick cross sections were cut from the paraffin blocks. These sections were mounted onto aminopropyl-triethoxysilane (APES)-coated glass slides for the immunohistochemical investigation of CK8, CK18, CK19, vimentin, desmin and laminin.

Immunohistochemistry (IHC)

A standard streptavidin – biotin immunoperoxidase technique was applied using an Ultravision Large Volume Detection System (Thermo Fisher Scientific Lab Vision Corporation, Fremont, CA, USA) to detect the IF proteins. Briefly, the sections were treated with 3% hydrogen peroxide (H_2O_2) in methanol for 15 min to block endogenous peroxidase activity. Subsequently, the sections were washed in PBS, placed in Tris-EDTA buffer (pH 9.0), heated in a water bath at 90°C for 30 min for antigen retrieval, and cooled for 20 min. The sections were then washed in PBS and treated with a blocking solution (Ultra V Block, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) for 5 min to prevent the nonspecific interference of immunoglobulins. Subsequently, the sections were incubated at 4°C overnight with the following antibodies: anti-cytokeratin 8 (C-43 clone, mouse monoclonal, Abcam, ab253, 1/100 dilution), anti-cytokeratin 18 (C-04 clone, mouse monoclonal, Abcam, ab668, 1/100 dilution), anti-cytokeratin 19 (A53-B/A2.26 clone, mouse monoclonal, Invitrogen, MA5-12663, 1/50 dilution), anti-vimentin (mouse monoclonal, Thermo Scientific, MS-129-R7, 1/200 dilution), anti-desmin (mouse monoclonal, Thermo Scientific, MS-376-S1, 1/200 dilution) and anti-laminin (rabbit polyclonal, Abcam, ab11575, 1/200 dilution). On the following day, the sections were washed in PBS and incubated for 20 min at room temperature with biotinylated secondary anti-rabbit or anti-mouse antibodies (Thermo Fisher Scientific Lab Vision Corporation, Ready-To-Use). After being washed in PBS, the sections were incubated with streptavidin peroxidase (Thermo Fisher Scientific Lab Vision Corporation, Fremont, CA, Ready-To-Use) for 20 min and washed in PBS once more. Finally, the sections were incubated with 3,3-diaminobenzidine tetrahydrochloride (DAB, TA-125-HD, Thermo Fisher Scientific Lab Vision Corporation, Fremont, CA, USA) for 5 min. The sections were then counterstained with Gill's hematoxylin for 3 min, washed under running tap water, dehydrated through an alcohol series, cleared in xylene, and mounted in Entellan (Merck).

The immunohistochemical staining specificity was checked using negative and positive control sections. As positive controls, slides of archival blocks of breast carcinoma were incubated with primary antibodies. The negative control reactions were performed by replacing the primary rabbit antibodies against CK8, CK18, CK19, vimentin, desmin and laminin with non-immune rabbit (Santa Cruz Biotechnology, sc-2027) or mouse (Santa Cruz Biotechnology, sc-2025) sera at similar concentrations or omitting the primary antibody step from the protocol. None of the negative controls showed immunostaining for any of the antibodies.

Tissue sections were examined by conventional light microscopy (Nikon-Eclipse 400) and evaluated for CK8, CK18, CK19, vimentin, desmin and laminin immunoreactivity. The sections were photographed with a digital camera (Nikon DSLR) using the NIS Elements Imaging Software-version 3.10.

Assessment of IHC staining results

Immunostaining was scored semiquantitatively for the expression of CK8, CK18, CK19, vimentin, desmin and laminin. The intensity of immunoreactivity for CK8, CK18, CK19, vimentin, desmin and laminin was scored as -, negative (no staining even at high magnification), +, weak (only visible at high magnification), ++, moderate (readily visible at low magnification), +++, strong (strikingly positive at low power magnification) staining (Sağsöz et al 2017). The expression of CK8, CK18, CK19, vimentin, desmin and laminin in the mammary gland was examined at 10X, 40X and 100X magnification, using a light microscope (E-400; Nikon, Tokyo, Japan) equipped with a DS-RI1 video camera (DS-U3, Nikon, Tokyo, Japan) during pregnancy, lactation and involution. The positively stained cells were evaluated and scored by two blind researchers (B.B. and H.S.) and the mean scores were calculated. Three random areas in each section, including at least 100 cells, were digitized by image analysis and computerized. As a result, namely, the alveolar epithelial cells, ductal epithelial cells, stromal cells, and myoepithelial cells of the rat mammary glands were assessed during pregnancy, lactation and involution (Table 1).

Results

CK8, CK18, CK19, vimentin, desmin and laminin were investigated immunohistochemically for their periodic localization and distribution patterns in the alveolar and ductal epithelial cells, stromal cells and myoepithelial cells of the mammary gland during pregnancy, lactation and involution. No immunolocalization was detected for any of the investigated parameters in the negative controls (Fig. 1). Differences were

Table 1. Intensity scores for CK8, CK18, CK19, Desmin, Vimentin, and laminin expression in rat mammary glands during pregnancy, lactation and involution.

Mammary Gland Periods		Tissue Layer of Mammary Gland	CK8	CK18	CK19	Desmin	Vimentin	Laminin
		Alveolar Epithelium	+++	N	N	N	N	+++
		Ductal Epithelium	+++	N	N	N	N	+++
		Addipose Tissue	N	N	N	N	N	N
	1st trimester	Stromal Cells	+++	+	N	+	N	+++
		Blood Vessels	N	N	N	++	N	+++
		Smooth Muscle	N	N	N	+++	N	+++
		Myoepithelium	N	N	N	N	N	+++
		Alveolar Epithelium	+++	+++	N	N	N	+++
Pregnancy		Ductal epithelium	+++	+++	N	N	N	+++
		Addipose Tissue	N	N	N	N	N	N
	2nd trimester	Stromal Cells	+++	++	N	+	N	+++
		Blood Vessels	N	N	N	++	N	+++
		Smooth Muscle	N	N	N	+++	N	+++
		Myoepithelium	N	N	N	N	N	+++
		Alveolar Epithelium	+++	+++	N	N	N	+++
		Ductal Epithelium	+++	+++	N	N	N	+++
		Addipose Tissue	N	N	N	N	N	N
	3rd trimester	Stromal Cells	+++	++	N	+	N	+++
		Blood Vessels	N	N	N	++	N	+++
		Smooth Muscle	N	N	N	+++	N	+++
		Myoepithelium	N	N	N	N	N	+++
		Alveolar Epithelium	+++	+++	N	N	++	+++
		Ductal Epithelium	+++	+++	N	N	++	+++
		Addipose Tissue	N	N	N	N	N	N
Lactation		Stromal Cells	+	N	N	+	N	+++
		Blood Vessels	N	N	N	+++	N	+++
		Smooth Muscle	N	N	N	+++	N	+++
		Myoepithelium	N	N	N	+++	N	+++
		Alveolar Epithelium	N	+++	N	N	++	+++
		Ductal Epithelium	N	+++	N	N	++	+++
		Addipose Tissue	N	N	N	N	N	N
Involution		Stromal Cells	+++	+	N	+	N	+++
		Blood Vessels	N	+	N	+++	N	+++
		Smooth Muscle	N	N	N	+++	N	+++
		Myoepithelium	N	N	N	+++	N	+++

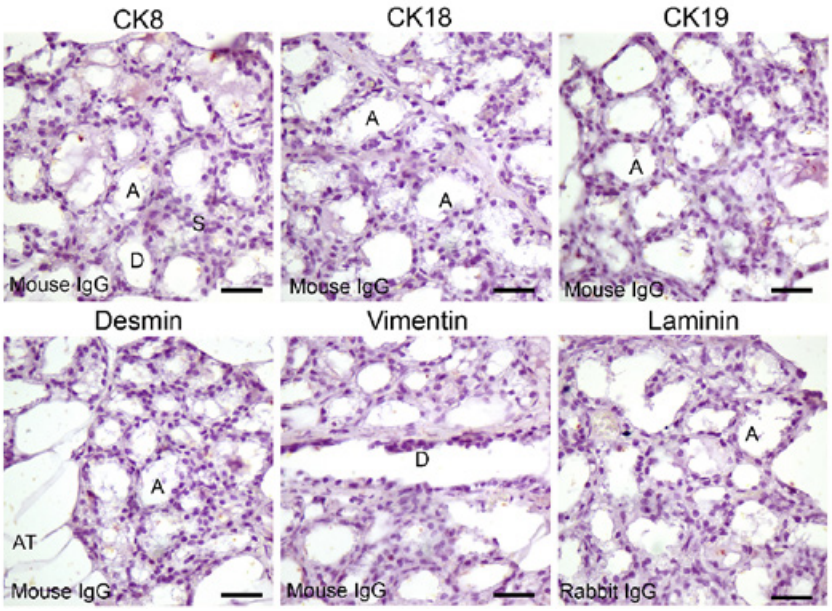

Fig. 1. Negative control produced using mouse IgG and rabbit IgG antibodies, resulted in no immunostaining for CK8 (involution), -18 (14th day of pregnancy), -19 (7th day of pregnancy), desmin (21st day of pregnancy), vimentin (lactation) and laminin (involution) in the rat mammary glands during pregnancy, lactation and involution. A, alveolus; D, ducts; S, stroma. Scale bars=25 µm.

observed in the spatial and temporal distribution of CK8, CK18, CK19, vimentin, desmin and laminin during pregnancy, lactation and involution (Table 1).

Mammary gland during pregnancy

CK8 expressions were observed to be relatively similar during all three trimesters of pregnancy. Strong CK8 expressions were detected particularly in the apical cytoplasm of the alveolar and ductal epithelial cells and in some stromal cells (Figs. 2A, 2B, 2C). On the 7th day of pregnancy, unlike CK8, CK18 was not expressed in the alveolar and ductal epithelial cells. Weak expression was observed only in some connective tissue cells. On the other hand, on days 14 and 21 of pregnancy, CK18 expression was strong in the alveolar and ductal epithelial cells and moderate in some stromal cells (Fig. 2D, 2E, 2F). Throughout pregnancy, CK19 was not expressed in any of the stromal cells or alveolar and ductal epithelial cells (Figs. 2G, 2H, 2I). Furthermore, the myoepithelium and fat cells, as well as the vascular endothelial and smooth muscle cells, were negative for CK8, CK18 and CK19 during pregnancy (Fig. 2A-2I). On the other hand, during all three trimesters of pregnancy, desmin expression was strong in the vascular smooth muscle cells, but weak in some stromal cells. However, desmin expression was not detected in the alveolar and ductal epithelial cells, myoepithelial and fat cells during pregnancy (Fig. 2J, 2K, 2L). Additionally, throughout the course of pregnancy, none of the tissue components contributing to the structure of the mammary gland showed vimentin expression (Fig. 2M, 2N, 2O). Throughout all three trimesters of pregnancy, laminin showed similar expressions, which were strong in the alveolar and ductal epithelia, myoepithelium, stroma, and vascular endothelial and smooth muscle cells. In contrast, the fat cells were observed not to express laminin (Fig. 2P, 2Q, 2R).

Mammary gland during lactation

During lactation, CK8 expression was strong in the alveolar and ductal epithelial cells, but rather weak in the stromal cells (Fig. 3A, 3B). While CK18 was strongly expressed in the alveolar and ductal epithelial cells, it was not expressed in the stromal and vascular endothelial cells (Fig. 3C, 3D). On day 7 of lactation, CK19 expression was not detected in the mammary gland (Fig. 3E, 3F). During the lactation period, the myoepithelial cells and fat cells did not express CK8, CK18 and CK19, and the mammary vasculature was also found to be negative for these cytokeratins (Fig. 3A-3F). During lactation, desmin was strongly expressed in the myoepithelial and vascular endothelial cells, and weakly expressed in some stromal cells. On the other hand, the alveolar and ductal epithelia and fat cells were devoid of desmin expression (Fig. 3G, 3H). On day 7 of lactation, there was moderate expression of vimentin in some alveolar and ductal epithelial cells, whereas none of the other tissue components contributing to the structure of the mammary gland expressed vimentin. (Fig. 3I, 3J). As to laminin, its expression was strong in the alveolar and ductal epithelial cells, myoepithelium, stromal cells, and vas-

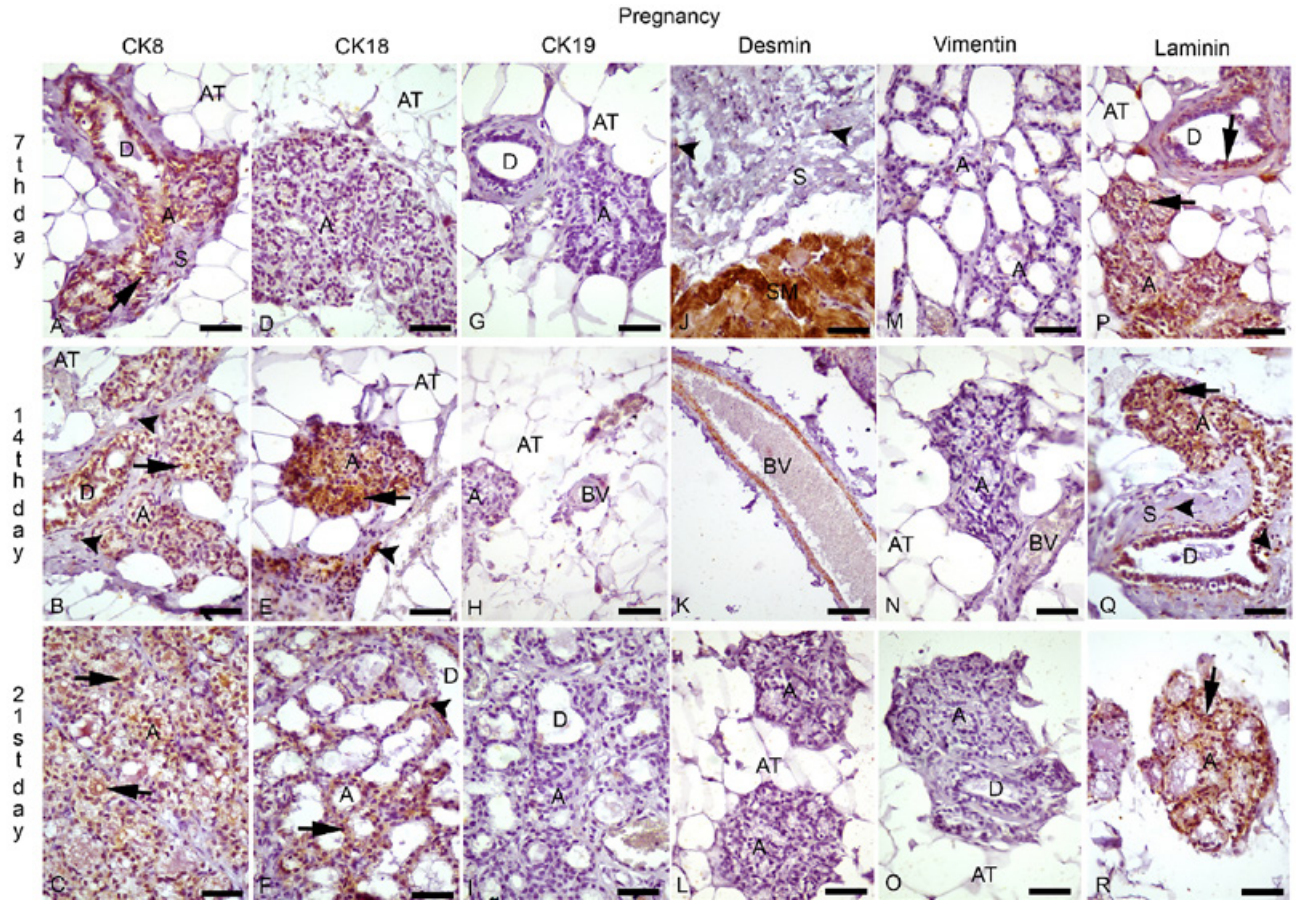

Fig. 2. Expression of IFs and laminin in the mammary glands of pregnant rats, immunohistochemical stain, diaminobenzidine as the chromogen. (A-C) localization of CK8 in alveolar and duct epithelial cells and stromal cells, (D-F) CK18 localization in alveolar and duct epithelial cells and stromal cells at 14 and 21 days of pregnancy, (G-I) CK19 negative appearance at all pregnancy periods, (J-L) localization of desmin in smooth muscles and in the media layer of blood vessels, (M-O) vimentin negative appearance at all pregnancy periods, (P-R) laminin localization in alveolar and duct epithelial cells and stromal cells at all pregnancy periods. A, alveolus; D, ducts; S, stroma; AT, adipose tissue; SM, smooth muscle cells; BV, blood vessel; arrows, positive alveolar and ductal epithelial cells; arrowheads, positive stromal and myoepithelial cells. Scale bars (A-C,E-G, I, J, K-R) 25=μm, (D,H,K)=50 μm.

cular endothelial and smooth muscle cells. The fat cells were observed not to express laminin (Fig. 3K, 3L).

Mammary gland during involution

While CK8 was strongly expressed by some stromal cells in the mammary gland, the alveolar and ductal epithelial cells, myoepithelium, fat cells and vascular endothelial and smooth cells were determined to be negative for CK8 (Fig. 4A, 4B). On day 7 of involution, CK18 expression was strong in the alveolar and ductal epithelial cells, and weak in the stromal cells and blood vessels. Similar to CK8, it was observed that CK18 was not expressed by the myoepithelial and fat cells (Fig. 4C, 4D). In the mammary gland, no CK19 expression was observed in the stromal, and alveolar and ductal epithelial cells, or the myoepithelium and blood vessels during involution (Fig. 4E, 4F). During this period, the expression of desmin was strong in the myoepithelium and vascular media, weak in some stromal cells, and absent in the alveolar and ductal epithelia and fat cells (Fig. 4G, 4H). Vimentin expression was moderate in the alveolar and ductal epithelial cells, but negative in the myoepithelial, stromal and fat cells, as well as in the blood vessels (Fig. 4I, 4J). On day 7 of involution, the alveolar and ductal epithelia, stroma, myoepithelium, and vascular endothelial and smooth muscle cells showed strong laminin expression. In contrast, the fat cells were devoid of laminin expression (Fig. 4K, 4L).

Discussion

The control of the expression of the genes which regulate hormonal and molecular factors in the mammary gland, is highly important for milk production in all species. In the development of the mammary gland, each cell type has a specific characteristic as regards the plasticity of the gland. As is the case with

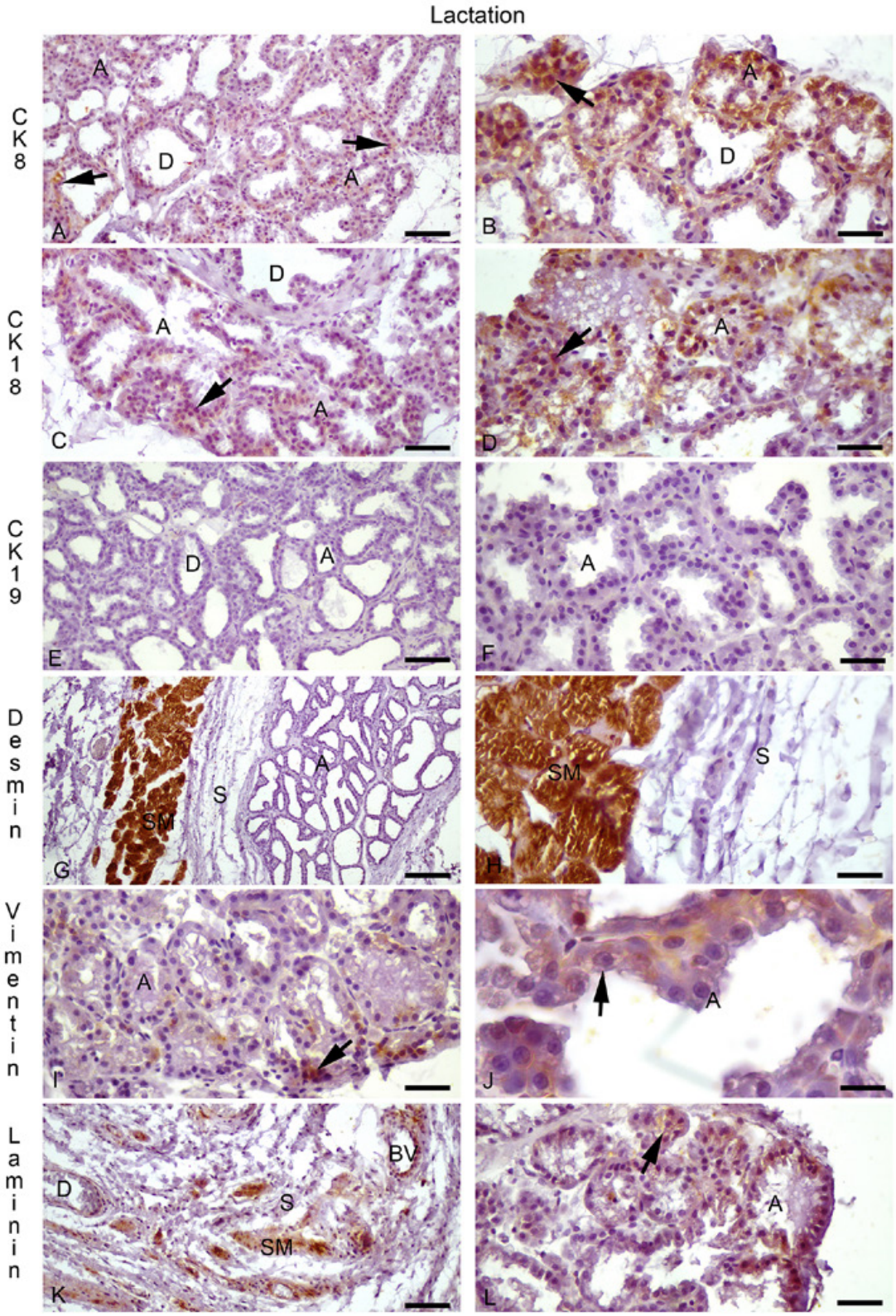

Fig. 3. Expression of IFs and laminin in rat mammary gland during lactation, immunohistochemical stain, diaminobenzidine as the chromogen. (A,B) CK8 localization in alveolar and duct epithelial cells, (C,D) CK18 localization in alveolar and duct epithelial cells and stromal cells, (E,F) negative appearance of CK19, (G,H) localization of desmin in smooth muscles, (I,J) weak vimentin expression in alveolar and duct epithelial cells, (K,L) laminin expression in alveolar and duct epithelial cells, stromal and smooth muscle cells and blood vessels. A, alveolus; D, ducts; S, stroma; SM, smooth muscle cells; BV, blood vessel; arrows, positive alveolar and ductal epithelial cells. Scale bars = (A,C,E,G)=50 μm, (B,D,F,H,K,L)=25 μm, (J)=6.25 μm.

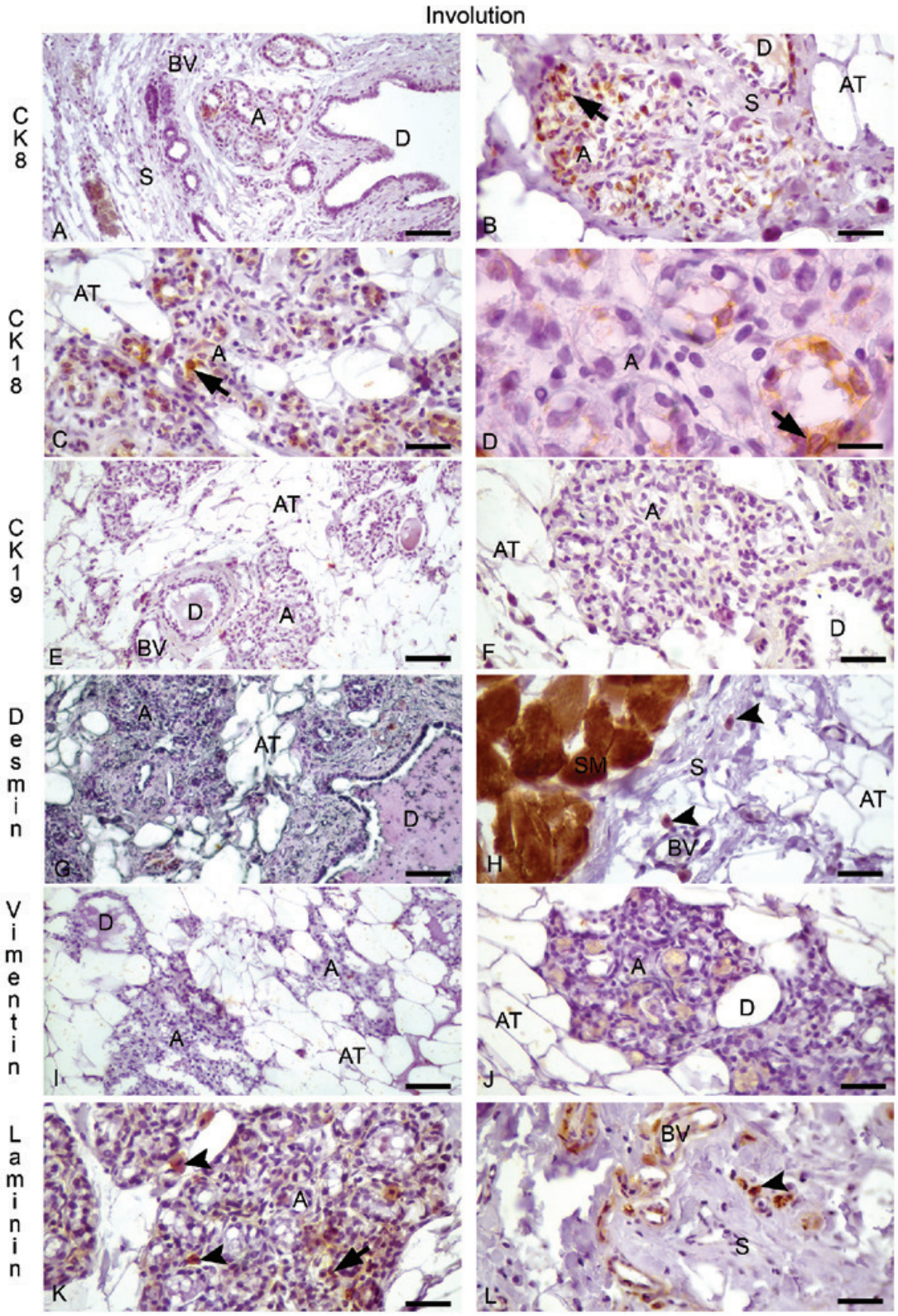

Fig. 4. Expression of IFs and laminin in rat mammary gland during involution, immunohistochemical stain, diaminobenzidine as the chromogen. (A,B) CK8 localization in alveolar and duct epithelial cells, (C,D) CK18 localization in alveolar and duct epithelial cells and stromal cells, (E,F) negative appearance of CK19, (G,H) localization of desmin in stmal and smooth muscles cell, (I,J) moderate vimentin expression in alveolar and duct epithelial cells, (K,L) laminin expression in alveolar and duct epithelial cells, stromal and smooth muscle cells and blood vessels. A, alveolus; D, ducts; S, stroma; AT, adipose tissue; SM, smooth muscle cells; BV, blood vessel; arrows, positive alveolar and ductal epithelial cells; arrowheads, positive stromal and myoepithelial cells. Scale bars = (A, E,G,I,K)=50 μm, (C,B, F,H,J,L)=25 μm, (D) 6.25=μm.

most glandular tissues, the adult mammary gland contains various types of cells, which interact to shape and activate the functions of the organ. Similarly to all eukaryotic cells, the cells that make up the mammary gland have a cytoskeleton composed of IFs and extracellular matrix proteins, which together form a fibrous network in the cytoplasm (Kohnen et al. 2000). In the present study, we demonstrated the expression of the intermediary filaments CK8, CK18 and CK19, vimentin, desmin and the connective tissue component laminin in the mammary tissue of adult rats. Our findings demonstrated that these components were localized to various tissue components, including the alveolar and ductal epithelia, stroma, myoepithelium and vascular endothelium and smooth muscle in the rat mammary gland throughout pregnancy, lactation and involution. The study findings also revealed spatial and temporal differences between these three periods. As is the case with other mammals, CK8, CK18, CK19, vimentin, desmin and laminin being expressed by certain cell groups in the mammary gland of the rat suggests that these factors contribute to both cytoskeleton formation and tissue integrity in this species.

The majority of literature reports on the expression of cytokeratins in humans and other mammals pertain to cases of breast cancer or other organ cancers. A literature review has shown that information and research available on the periodic and cellular localization of these factors in relation to the embryonic development of the mammary gland or hormone-induced cellular changes during pregnancy, lactation and involution, are scarce. Reports indicate that CK8 is one of the cytokeratins first expressed during embryogenesis, which starts with expression in organs lined by simple epithelium (i.e. liver, pancreas, kidneys) (Owens and Lane 2003) and continues with expression in organs lined by mixed epithelia (i.e. mammary gland, lungs) (Franke et al. 1981). It has been indicated that, in humans, CK8 is expressed by the luminal epithelial cells of the mammary gland, which serve as a differentiation site (Buhler and Schaller 2005). In mice, CK8 has been determined to be localized to the simple alveolar and luminal epithelia during the development of the mammary gland and to display a heterogenous expression pattern (Santagata et al. 2014). In cows, CK8 has been shown to be expressed by the alveolar and ductal epithelia and some stromal cells of the mammary gland during late lactation and involution (Alklay et al. 2022). Similarly to these reports, the present study showed that CK8 was strongly expressed by the alveolar and ductal epithelial cells of the rat mammary gland during all three trimesters of pregnancy and the lactation period. On the other hand, the alveolar and ductal epithelial cells of the rat mammary gland were observed to be negative for CK8 during the involution period. Similarly to cows, rats displayed moderate or weak CK8 expression in the stromal cells of the mammary gland during pregnancy and lactation, and strong CK8 expression in the mammary stroma during involution. In contrast to reports in humans and mice, it has been determined that, in rats, myoepithelial and fat cells as well as vascular endothelial and smooth muscle cells are negative for CK8 during pregnancy, lactation and involution. Cytokeratins are intermediate filament proteins found in most epithelial cells. Among cytokeratins, CK8/CK18 expression has been demonstrated in luminal epithelial cells of the mouse mammary gland (Buhler and Schaller 2005). CK8 has already been shown to be determined during embryonic mammary gland development in the mouse (Franke et al. 1981). In the mature mammary gland, luminal and ductal epithelial cells have been reported to express high levels of CK8 (Buhler and Schaller 2005). In this study, it was shown that CK8 expression was localized in luminal and ductal epithelial cells during gestation and lactation periods and in stromal cells during the involution period in the rat mammary gland. These results suggest that CK8 may play a role in cell differentiation and cytoskeleton formation during pregnancy, lactation and involution in the rat mammary gland.

Some human studies have reported that while CK18 was not expressed in the cells of the normal mammary tissue, its expression was strong in breast tumors (Abd El Rehim et al. 2004). On the other hand, other human studies have indicated that, in the normal mammary gland, CK18 was expressed at varying levels in the alveolar and ductal epithelial cells (Böcker et al. 2002). Furthermore, in ruminants, CK18 was shown to be expressed at varying levels in the alveolar and ductal epithelia and myoepithelium of normal mammary tissue (Boutinaud et al. 2015). Varying levels of CK18 expression have been reported in some stromal cells as well as in the alveolar and ductal epithelia in the mammary gland of cows during late lactation and involution (Alklay et al. 2022). Furthermore, strong in vitro CK18 expression has been determined in mouse and cattle epithelial cells (Petridis and Fthenakis 2019). Similarly to previous reports in humans, mice (Petridis and Fthenakis 2019) and ruminants (Abd El Rehim et al. 2004, Petridis and Fthenakis 2019, Alklay et al. 2022) the present study demonstrated varying levels of CK18 expression in some stromal cells as well as the alveolar and ductal epithelial cells of the rat mammary gland during pregnancy, lactation and involution. Moreover, contrary to the report in ruminants (Boutinaud et al. 2015), we observed no CK18 expression in the myoepithelial and fat cells of the rat mammary gland during the three investigated periods. In rats, CK18 expression

was negative in the mammary vasculature throughout pregnancy and lactation and was weak during involution. Based on the above information, these results suggest that CK18 may play a role in cell differentiation and cytoskeleton formation during pregnancy, lactation and involution in the rat mammary gland.

Complicated cellular processes, which occur in the normal mammary gland during pregnancy, lactation and involution (epithelial and stromal cell differentiation), in general, can be detected based on the expression of certain cytokeratins (Coulombe and Omary 2002). Mammary epithelial cells can be identified according to their pattern of expressing intermediate filaments. However, as the expression of specific cytokeratins may differ between the ductal epithelium and alveolar epithelium, further differences in localization may be observed between species and the different regions of the mammary gland (Dontu and Ince 2015). In this context, the biological role of heterogenous cytokeratin expression in normal and neoplastic mammary gland tissues has not yet been fully understood (Coulombe and Omary 2002, Ontsouka et al. 2016). Cytokeratin filaments are involved in the establishment of the mechanical scaffold required by epithelial cells. In any case, they regulate tissue or organ morphogenesis and cell differentiation. Cytokeratins display various molecular and functional tissue expressions, depending on the type and differentiation characteristics of epithelial cells. Thus, cytokeratins provide data on various cytogenetic changes in tissues (Abd El Rehim et al. 2004). Among cytokeratins, which are a family of intermediate filaments, CK18 is described as being the member most commonly found in tissues and organs, and is indicated to be present generally together with CK8. Both of these cytokeratins are expressed by simple epithelial cells and are described as important markers for alveolar and ductal epithelial cells (Abd El Rehim et al. 2004, Böcker et al. 2002). In view of these data, varying levels of CK8 and CK18 expression having been determined in the different cell components of the rat mammary gland during pregnancy, lactation and involution suggests that these cytokeratins could have similar functions in the rat mammary gland during all three of the investigated periods.

CK19 is known to be an important marker for alveolar epithelial cells (Ontsouka et al. 2016). CK19 expression is mostly observed in stem cells, cells with high differentiation capacity, and cells of epithelial origin with high plasticity, such as tumor cells (Mujyambere et al. 2018). While in situ human studies have reported that, in the normal mammary tissue, CK19 was localized to the epithelial cells, they also indicated that CK19 expression lacked specificity (Bartek et al. 1985). Multiple studies have reported CK19 expression as being absent in the cells of the normal mammary tissue, but strong in malignant tumor masses (Petersen et al. 1998). It has also been reported that, alveolar and ductal epithelial cells of the human and mouse mammary glands expressed CK19 at varying levels (Abd El Rehim et al. 2004). In particular, extensive CK19 expression has been reported in the terminal ductal epithelium of the mammary gland of nulliparous mice (Anbazhagan et al. 1998, Smalley et al. 1998). Ontsouka et al. (2016) demonstrated that, in cows, CK19 levels were either very low or undetectable in the primary (epithelial) cells of the mammary gland during pregnancy, lactation and involution. However, these researchers also determined that the cultures of cells from these periods were positive for CK19 expression (Ontsouka et al, 2016). Alklay et al. (2022) reported the absence of CK19 expression in the bovine mammary gland during late lactation and involution. In the present study, in agreement with previous studies (Petersen et al. 1998, Alklay et al. 2022), CK19 expression was not detected in any of the tissue components of the rat mammary gland during pregnancy, lactation and involution. No periodic CK19 expression having been detected in the rat mammary gland in the present study clearly shows the complicated nature of mammary cells, and suggests that analyses based solely on the expression of cell type markers could be misleading and thus, risky. However, despite the results of our study, it is still valid to consider CK19 as a marker for the identification of mammary gland epithelial cells as well as cultures of cells from the mammary gland.

In cultures of human mammary gland cells, both myoepithelial and stromal cells have been reported to react positively for vimentin, whilst epithelial cells have been indicated as being vimentin-negative (Mark et al. 1990). In addition to the report of vimentin being expressed at varying levels in the myoepithelial and stromal cells of the normal human mammary gland, it has also been indicated that vimentin is localized to the vascular endothelial cells (Petre et al. 2016). Vimentin expression has been shown to be weak in the mammary gland of mice, and to eventually cause impaired development of the mammary alveoli and ducts as well as a decrease in their capability to regenerate (Peuhu et al. 2017). In the normal mammary gland of dogs, both the interstitial tissue and the peripheral cells of the ducts and acini (most probably myoepithelial cells) were reported as being vimentin-positive (Hellmén and Lindgren 1989). Similarly, there are reports indicating the detection of vimentin-positive reactions in the myoepithelial cells of the rat mammary gland during various periods (Warburton et al. 1985). Marettová and Maretta (2018) reported vimentin expression in the fibroblasts (myofibroblasts) of the

connective tissue of the interalveolar and interlobular septa, as well as in some alveolar epithelial cells, some free cells such as lymphocytes and macrophages in the septal connective tissue, and vascular endothelial cells in the mammary gland of lactating cows. Vimentin was also reported to be expressed in some cells of the ovine mammary stroma (Peuhu et al. 2017, Marettová and Maretta 2018). In contrast to the studies referred to above (Warburton et al. 1985, Hellmén and Lindgren 1989, Petre et al. 2016, Peuhu et al. 2017, Marettová and Maretta 2018), the present study showed that vimentin expression was not present in all tissue components of the rat mammary gland during pregnancy and lactation, vimentin was moderately expressed in the alveolar and ductal epithelial cells during involution, and was absent in the myoepithelium, stroma, fat cells and vasculature. This was attributed to vimentin being actively involved in the cellular renewal and return to normal of the rat mammary gland rather than its cellular development and functional maintenance during pregnancy and lactation.

Desmin interacts with other proteins to form the cytoskeletal network, and enables the maintenance of cell integrity, force transmission and mechanochemical signals (Goldfarb and Dalakas 2009). Desmin expression has been demonstrated in the thick interlobular connective tissue septa, smooth muscle cells of the vascular media layer, and myoepithelial cells in the cow mammary gland. On the other hand, it has been reported that desmin is not expressed in the normal mammary gland or mammary carcinomas of humans and some other species (Mou et al. 2013). Additionally, previous research on desmin suggests that it affects muscle tissue. Furthermore, several studies have shown that desmin is not localized to the epithelial and stromal cells of the mammary gland (Mou et al. 2013). In the present study, it was determined that, in the rat mammary gland, desmin was expressed by myoepithelial cells, some muscular structures and the smooth muscle cells of the vascular media during pregnancy, lactation and involution. However, desmin was not detected in the alveolar and ductal epithelia, stroma and fat cells of the rat mammary gland. The presence of desmin in the myoepithelial cells of the rat mammary gland during all three of the investigated periods suggests its possible role in the expulsion of milk from the alveoli and the continuity of mechanochemical signals. Furthermore, the presence of desmin expression in some smooth muscle structures and blood vessels in the mammary gland during pregnancy, lactation and involution suggests that it may be involved in mammary gland development and remodeling.

It has been reported that, during pregnancy, laminin expression is mainly confined to the stromal cells surrounding the mammary alveoli and ducts. The same report indicates the existence of no difference between the trimesters, the presence of strong expression in the connective tissue, developing blood vessels, skeletal muscle and stromal cells supporting the fat tissue, and the absence of expression in epithelial cells (Keely et al. 1995). Reports have indicated high levels of laminin expression in the basal and luminal cells of the mouse mammary gland (Lim et al. 2010, Bach et al. 2017). Based on the expression of laminin concentrating largely to the stromal cells surrounding the small ducts and developing alveoli, many researchers have suggested that laminin may contribute to in vitro mammary morphogenesis (Keely et al. 1995). Hohenester et al (2013) determined that laminins, together with collagen IV, have a major role in the adhesion of mammary epithelial cells to the basal membrane (Hohenester et al. 2013). Several researchers have attributed the expression of laminin in epithelial cells to its possible role in the inhibition of mammary alveolar development (Li et al. 2005). In the present study, we determined that, in the rat mammary gland, laminin expression was similar during all three trimesters of pregnancy, lactation and involution, and strong in the alveolar and ductal epithelia, myoepithelium, stromal cells, and vascular endothelial and smooth muscle cells. No laminin expression was detected in the fat cells. Strong laminin expression having been determined in the rat mammary gland was attributed to the possible role of laminin in the development of the rat mammary gland during pregnancy and lactation. Furthermore, strong laminin expression during the involution period suggested that laminin could be involved in alveolar, ductal and stromal repair.

In conclusion, the present study demonstrated either the absence or the presence of varying levels of CK8, CK18, desmin, vimentin, and laminin expression in the various tissue components of the rat mammary gland during pregnancy, lactation and involution with periodic differences, and the absence of mammary CK19 expression during all three periods. These results showed that laminin and other intermediate filaments, rather than CK19, could have a role in the development and repair of the rat mammary gland. In view of their described functions, we suggest that intermediate filaments could be effective in the development of the mammary tissue, as well as the production and expulsion of milk. Moreover, the detection of higher levels of IFs in cancerous tissues, compared to normal tissues, also suggests that IFs could be involved in tumor development, and that the complete isolation of these filaments in the future could enable their use for treatment purposes.

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