Published in final edited form as:

Domest Anim Endocrinol. 2011 November ; 41(4): 185–194. doi:10.1016/j.domaniend.2011.06.005.

Expression of gap junctional connexin proteins in ovine fetal ovaries: Effects of maternal diet

A.T. Grazul-Bilska*, K.A. Vonnahme, J.J. Bilski, E. Borowczyk, D. Soni, B. Mikkelsen, M.L. Johnson, L.P. Reynolds, D.A. Redmer, and J.S. Caton

Department of Animal Sciences, Center for Nutrition and Pregnancy and Cell Biology Center, North Dakota State University, Fargo, ND, USA

Abstract

Gap junctions have been implicated in the regulation of cellular metabolism and the coordination of cellular functions during growth and differentiation of organs and tissues, and gap junctions play a major role in direct cell-cell communication. Gap junctional channels and connexin (Cx) proteins have been detected in adult ovaries in several species. Furthermore, it has been demonstrated that several environmental factors including maternal diet may affect fetal organ growth and function. To determine if maternal diet impacts expression of connexin (Cx) 26, 32, 37 and 43 in fetal ovaries, sheep were fed a maintenance (M) diet with adequate (A) selenium (Se) or high (H) Se levels from 21 days before breeding to day 132 of pregnancy. From day 50 to 132 of pregnancy (tissue collection day), a portion of the ewes from ASe and HSe groups was fed restricted (R; 60% of M) diet. Sections of fetal ovaries were immunostained for the presence of connexins followed by image analysis. All four connexins were detected, but the distribution pattern differed. Cx26 was immunolocalized in the oocytes from primordial, primary, secondary and antral follicles, in granulosa and theca layers of secondary and antral follicles, stroma and blood vessels; Cx32 was in oocytes, granulosa and theca cells in a portion of antral follicles; Cx37 was on the borders between oocyte and granulosa/cumulus cells of primordial to antral follicles, and in endothelium; and Cx43 was on cellular borders in granulosa and theca layers, and between oocyte and granulosa/cumulus cells of primordial to antral follicles. Maternal diet affected Cx26 and Cx43 expression; Cx26 in granulosa layer of antral follicles was decreased ($P<0.01$) by HSe in M and R diets, and Cx43 in granulosa layer of primary and granulosa and theca of antral follicles was increased ($P<0.05$) by M diet with HSe. Thus, connexins may be differentially involved in regulation of fetal ovarian function in sheep. These data emphasize the importance of maternal diet in fetal growth and development.

Keywords

connexins; gap junctions; fetal ovaries; maternal diet; developmental programming

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*Corresponding author: Department of Animal Sciences, North Dakota State University, Fargo, ND 58108, USA. Tel. +1 701-231-7992; fax: +1 701-231-7590. Anna.Grazul-Bilska@ndsu.edu.

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1. Introduction

Fetal ovaries represent a type of tissue that expresses high tissue growth and differentiation, which are controlled by factors of fetal and maternal origin [1–4]. In fact, numerous factors of fetal and maternal origin, including nutrition, FSH, LH, estrogens, activin, c-kit with its ligand stem cell factor, enzymes controlling steroidogenesis, growth differentiation factor 9, epidermal growth factor and many others, may affect the growth, development, and physiology of the fetal and postnatal ovarian function of mammals [5–10]. Additionally, our previous study demonstrated that maternal diet affected fetal body and ovarian weight, and fetal ovarian cell proliferation in sheep [4].

Regulation of organ growth and function can be mediated by contact-independent and contact-dependent mechanisms [11]. Contact-dependent cellular interactions are mediated by gap junctional pathways which facilitate and regulate the exchange of regulatory molecules [11]. Gap junctions have a hexameric structure known as a connexon that is composed of specific gap junctional proteins termed connexins [11–16]. It has been well established that connexins not only form gap junctional channels, but also have additional functions including signal transduction, secretory function, paracrine signaling and control of growth and development of organs and tissues in physiological and pathological conditions [17–22].

Gap junctions and/or gap junctional proteins or mRNA are expressed in the adult ovaries of several species [11,23–30]. However, for fetal ovaries, expression of mRNA for several connexins and Cx43 protein has been only demonstrated in mice [31,32]. In ovaries, connexins exhibit a unique pattern of expression that can be metabolically, hormonally or developmentally regulated [11,23,30].

We hypothesized that Cx26, Cx32, Cx37 and Cx43 proteins are expressed in fetal ovaries, and that expression of these connexins will be affected by maternal diet. Therefore, the objective of this experiment was to immunolocalize Cx26, Cx32, Cx37 and Cx43 in ovarian compartments, and to determine if maternal plane of nutrition including level of energy in diet (e.g., maintenance [M] vs. restricted [R] diet) and Se level (e.g., adequate ([A] vs. High [H] concentration) impacts expression of connexin proteins in fetal ovaries in sheep. We choose to determine expression of four listed above connexins since 1) all of them are expressed in adult ovaries in several species, 2) these connexins may play a specific role in ovarian function, and 3) expression of these connexin may depend on maternal factors (as described above, and in the Discussion section).

1. Materials and Methods

1.1. Animals and Treatments

The Institutional Animal Care and Use Committee at NDSU approved all animal procedures in this study. Animal feeding, maintenance and management are described in detail by Grazul-Bilska et al. [4]. Briefly, following breeding, pen-fed basal diet (2.04 kg/ewe daily) ewes were assigned randomly to either an A or H dietary Se treatment. In addition, ewes were fed 100 g/day of a control pellet that contained 0.3 ppm ASe or a high-Se pellet balanced to contain 47.5 ppm Se provided as Se-enriched yeast (Sel-Plex, Alltech, Nicholasville, KY, USA). This approach provided 6 µg/kg of ewe body weight in the ASe and 80 µg/kg of ewe body weight in the HSe treatments, respectively. The ASe and HSe pellets were formulated using similar ingredients to maintain similar concentrations of metabolizable energy (ME), crude protein, acid detergent fiber, neutral detergent fiber, Ca, and P. The approach by which dietary Se was supplemented to pregnant, primigravid ewes has been used previously by our laboratory [4, 33].

On day 50 of gestation, ewes within each Se treatment were stratified by average breeding date and weight, and assigned to distinct planes of nutrition treatments. Ewes were offered diets that were balanced to meet either 100% (M) or 60% (R) of predicted ME requirements of pregnant ewe lambs [34], and were kept and fed in individual pens. The dietary restriction treatment was accomplished by reducing intake of restricted ewes to 60% of control treatment and therefore represents a global nutrient reduction. The plane of nutrition treatments was applied from day 50 to 132 of pregnancy, which resulted in 4 distinct treatment combinations designated by the following: MASE ($n=8$ ewes), MHSe ($n=8$), RASE ($n=10$) and RHSe ($n=6$). Only ewes with female singleton fetuses were included in this study.

1.2. Tissue Collection and Immunohistochemistry

On day 132 ± 1 of the pregnancy, fetal ovaries were collected. One ovary from each fetus was fixed in Carnoy's solution, and after dehydration, ovaries were embedded in paraffin. Ovaries ($n = 6-10/\text{treatment}$) were sectioned (one section/ovary along the longitudinal axis) at $5 \mu\text{m}$ and mounted onto a glass slide.

Detection of connexins in Carnoy's fixed tissues was performed as previously described [23, 28, 29]. Briefly, ovarian tissue sections were deparaffinized, rehydrated, and incubated with 3% H_2O_2 in methanol to eliminate endogenous peroxidase activity. Then, sections were rinsed several times in PBS containing Triton-X100 (0.3%, vol/vol) and treated for 20 min with PBS containing normal horse serum (3%, vol/vol; ABC kit, Vector Laboratories, Burlingame, CA, USA) to block nonspecific binding of antibodies. Sections were incubated overnight at 4 C in PBS containing a primary rabbit polyclonal antibody against Cx26, Cx32 (1:50; Zymed Laboratories Inc., San Francisco, CA, USA), Cx37 (1:50; Alpha Diagnostics, San Antonio, TX, USA) or Cx43 (1:500; [23]). Primary antibody was detected using biotin-labeled anti-rabbit secondary antibody and the ABC method (Vector Laboratories). For color development of Cx26, Cx37 and Cx43, SG substrate was used as described before [23]. The Cx32 primary antibody was detected using fluorescein isothiocyanate conjugated secondary antibody (Zymed Laboratories; [22]), and cell nuclei were stained with propidium iodide (500 ng/ml; Molecular Probes, Eugene, OR, USA; [35]). For controls, the primary antibody was replaced with rabbit serum. Each primary antibody was specific for a specific Cx, as described in the information sheet by manufacturer (<http://www.invitrogen.com/search/global/searchAction.action?query=connexinantibody&resultPage=1&resultsPerPage=15&autocomplete=true>), and confirmed in our laboratory using Western immunoblot analysis and immunoneutralization [23,24, Grazul-Bilska and Johnson, unpublished].

1.3. Image analysis

For all ovaries, images of stained sections were taken for each of the four types of follicles (e.g., primordial, primary, secondary and antral), for stromal tissue and blood vessels within the medulla and hilus not containing follicles (total 5–15 images/ovary) using Eclipse E600 Nikon microscope or Axio Observer Zeiss microscope with a digital camera. The images were then used for quantitative image analysis using the Image Pro-plus software (Media Cybernetics Inc., Silver Spring, MD, USA) to determine the proportion (%) of area stained positively for a specific connexin out of the total area of granulosa or theca layer. Image analysis was performed for Cx26 and Cx43 in primary, secondary and/or antral follicles. Image analysis quantification was not performed for Cx26 in oocytes for any follicle type due to heterogeneous distribution and in theca layer of antral follicles due to relatively weak and heterogeneous distribution, and for Cx32 and Cx37 due to the heterogeneous distribution within the follicles. The number of primordial, primary, secondary and antral

follicles analyzed for Cx26 and Cx43 expression using image analysis is presented in Table 1.

1.4. Statistical Analyses

Data were analyzed as a completely randomized design of treatments using PROC GLM (SAS Inst. Inc. Cary, NC 2010). The model contained effects for plane of nutrition (M and R), with different two levels of Se (ASe and HSe), the plane of nutrition x Se interactions, and ewe was used as a covariate. When the F-test was significant ($P < 0.05$), differences among means were separated by using the least square means procedure [37]. Means were considered different when $P < 0.05$ unless otherwise stated. Data are expressed as mean $\pm$ SEM.

2. Results

All four connexins were detected in fetal ovaries, but the pattern of localization of these connexins differed (Fig. 1–3; Table 2). Connexin 26 was immunolocalized in the cytoplasm of oocytes from primordial, primary, secondary and antral follicles, on the cellular borders and/or in the cytoplasm in granulosa cells of secondary and antral follicles, theca layer of antral follicles, in stroma and blood vessels (Fig. 1). Staining of Cx26 in granulosa layer of secondary follicles and theca layer of antral follicles seemed to be weaker than in granulosa layer in antral follicles (Fig. 1). Connexin 32 was expressed as punctuate staining in oocytes, granulosa and thecal cells of approximately 20% of antral follicles (Fig. 2A). Connexin 37 was expressed as linear and punctate staining on the borders between oocyte and granulosa/cumulus cells of primordial, primary, secondary and antral follicles, and as punctate and cytoplasmic staining in endothelium (Fig. 2B–D). Connexin 43 was expressed as punctate staining on the cellular borders and cytoplasm in the granulosa layer of primordial, primary, secondary and antral follicles, in the theca layer of antral follicles, and on borders between the oocyte and granulosa/cumulus cells (Fig. 3).

Expression of Cx26 in granulosa cells of secondary follicles was not affected by nutritional treatments (data not shown), but Cx26 expression in granulosa cells of antral follicles was less ($P < 0.01$) in MHSe and RHSe groups than in MASE group (Fig. 4A). Overall, Cx26 expression in granulosa cells of antral follicles was greater ($P < 0.0001$) than in granulosa cells of secondary follicles (0.35 ± 0.04 vs. $0.12 \pm 0.01\%$).

Expression of Cx43 in the granulosa layer of primary follicles was greater ($P < 0.05$) in the group fed M diet with HSe than in groups fed M or R diets with ASe (Fig. 4B). For secondary follicles, Cx43 expression was similar in all treatment groups (data not shown). For antral follicles, Cx43 expression in granulosa and theca layers was greater ($P < 0.01$) in the group fed M diet with HSe than in any other treatment group (Fig. 4C). Overall, Cx43 expression was greater ($P < 0.001$) in granulosa than in the theca layer of antral follicles (1.5 ± 0.1 vs. $0.6 \pm 0.05\%$), and expression of Cx43 in granulosa cells was greater ($P < 0.001$) in antral than in secondary or primary follicles (1.5 ± 0.1 vs. 0.6 ± 0.05 or $0.5 \pm 0.06\%$, respectively).

3. Discussion

The results of this experiment show that Cx26, Cx32, Cx37 and Cx43 are expressed in fetal ovaries and that distribution of these connexin within ovarian compartments differs. In addition, maternal dietary restrictions and/or level of Se in the diet differentially affected Cx26 and Cx43 expression depending on fetal ovarian compartment. This suggests that maternal plane of nutrition is involved in the control of gap junction expression. These observations are novel, since very limited information is available concerning the expression

of connexin proteins and regulation of their expression in fetal ovaries. In addition, these data emphasize that maternal diet may have the major impact on normal fetal ovarian growth and development, which is a central concept of fetal/development programming also known as developmental origins of health and disease [38,39].

The pattern of Cx26 localization in the oocytes from primordial and primary follicles, granulosa and thecal layers of secondary and antral follicles, in stromal tissues and blood vessels in fetal ovaries resembled the pattern observed in adult ovine ovaries [23]. However, in contrast to adult ovine and bovine ovaries, in ovine fetal ovaries Cx26 was not expressed in surface epithelium and it was expressed in oocytes of secondary and antral follicles [23, 24]. Moreover, Cx26 seems to be developmentally regulated in fetal and adult ovaries since Cx26 expression was the greatest in antral follicles in this study, and in large antral follicles in adult cows [24]. The presence of Cx26 in the granulosa layer of secondary and granulosa and theca layers of antral follicles, stromal tissue and blood vessels suggests its role in the regulation of follicular development, and stromal and blood vessel function in fetal ovaries. In addition, Cx26 is likely involved in maintenance of oocyte health, since decreased Cx26 expression in cumulus oocyte complex was associated with a decreased quality of oocytes in the diabetic mouse model [40]. However, in the present study, Cx26 was expressed in cumulus cells only in a small portion of the antral follicles present in fetal ovaries. Therefore, additional studies should be undertaken to determine what role Cx26 may have in maintenance of oocyte health in fetal ovaries in sheep.

Connexin 32 was expressed only in the portion of antral follicles in fetal ovaries in the present study. In adult healthy ovine follicles, Cx32 protein was not detected in oocyte, granulosa and theca cells [23]. But, Cx32 protein was detected in ovarian stromal tissues, blood vessels, corpora lutea, oocyte and/or atretic follicles in adult sheep, cows or mice [23,24,41,42]. In cows, Cx32 expression was associated with follicular atresia, and it was speculated that Cx32 may serve as a marker of atresia [24]. Based on morphology (e.g., presence of pyknotic nuclei) and TUNEL staining [4], we could not detect any atretic follicles in fetal ovaries in the present study. We hypothesize that expression of Cx32 in some follicles may indicate the initiation of follicular atresia. However, Cx32 may not be critical for ovarian function, since it has been demonstrated that Cx32 knockout mice maintain normal reproductive function [43]. Thus, a role of Cx32 in regulation of fetal ovarian function remains unclear and may be of limited importance.

In the present study, Cx37 protein was uniformly and consistently distributed on oocyte-cumulus/granulosa borders from primordial to antral follicles and in endothelium. Similarly, in adult ovine ovaries, Cx37 protein was detected on the oocyte/cumulus border and in endothelial cells [29]. In addition, Cx37 was present in granulosa cells of adult ovine and bovine antral follicles, and murine oocytes [25,29,40,44]. For the mouse, it has been clearly demonstrated that Cx37 is critical for normal oogenesis/folliculogenesis [45,46]. Localization of Cx37 on the border between oocyte and cumulus/granulosa cells in all follicular stages in fetal ovaries in the present study, suggests that Cx37 may be as important for ovine oocyte/follicle development as it has been shown for mice.

Connexin 26 and Cx37, but not Cx32 or Cx43, were expressed in blood vessels in fetal ovaries in this study. In fact, a dense network of blood vessels in the medulla and hilus and a less dense network in the cortex are present in ovine fetal ovaries [4]. It has been demonstrated that several connexins including Cx37, Cx40, Cx43 and Cx45 are involved in the regulation of vascular function and angiogenesis in several organs in adults [47–49]. It seems that type of connexin expressed in blood vessels in fetal ovaries differs from adult organs, since in fetal ovaries we detected the presence of Cx26 but not Cx43. This difference

is likely due to the early developmental stage of fetal ovaries. Therefore in fetal ovaries, Cx26 and Cx37 are likely involved in regulation of blood vessel function and angiogenesis.

Connexin 43 was exclusively expressed in granulosa and theca layer in all follicle types, and its expression level increased from the primary to antral stage of follicular development in the present study. Similarly, Cx43 was detected during fetal ovarian development in the mouse [32]. In addition, a similar spatio-temporal pattern of Cx43 expression was observed for adult ovaries in several species [23–25,44]. Thus, the Cx43 expression pattern suggests that Cx43 is involved in the regulation of folliculogenesis in ovine fetal ovaries. In addition, it has been clearly demonstrated that level of Cx43 expression in cumulus cells may serve as a marker of oocyte quality in humans [50], and that Cx43 is directly associated with steroidogenesis in the ovine ovary [22]. The importance of Cx43 for follicular development was emphasized in a mouse knockout model showing a lack of development beyond primary follicles [30,51]. Thus, it is reasonable to postulate that in fetal ovaries similar to adult ovaries, Cx43 plays a major regulatory role during folliculogenesis.

In this study, maternal diet affected expression of Cx26 and Cx43 proteins in fetal ovaries; Cx26 in antral follicles was decreased by HSe level in M diet, and Cx43 in primary and antral follicles was increased by HSe level in M but not in R diet. We hypothesize that these differences may be due to a different level of expression and distribution of each connexin, and to the different functions of Cx26 vs. Cx43 in fetal ovaries. Differential effects of maternal diet on fetal ovarian weight, delayed ovarian growth and development, and altered ovarian cell proliferation and apoptosis have been reported for sheep [4,5,52–55]. Recently, we clearly demonstrated that maternal dietary restrictions and/or HSe level decreased cellular proliferation in primordial, secondary and/or antral follicles, stromal tissues and blood vessels in fetal ovaries [4]. Interestingly, the enhanced Cx43 but decreased Cx26 expression observed in the present study in primary and/or antral follicles in the group fed M diet with HSe corresponds to decreased cellular proliferation in the same compartments of fetal ovaries from the same nutrition-treatment group [4]. Therefore, we hypothesize that changes in expression of Cx26 and Cx43 may be a part of a compensatory mechanism preventing an additional decrease of cell proliferation and maintaining follicle integrity in fetal ovarian follicles when cell proliferation is reduced by HSe in the diet. Furthermore, it seems that there are different mechanisms of regulation of Cx26 and Cx43 expression by maternal diet. In addition, Cx43 is recognized as an important regulator of cell and tissue growth [11, 17], and it has been demonstrated that Se is involved in regulation of several cellular functions such as cell proliferation, angiogenesis and/or gap junction function in many organs [33,56–61]. Thus, these data indicate that Se is involved in the regulation of Cx26 and Cx43 expression and/or function. However, additional studies should be undertaken to determine the mechanism of the effects of the level of energy and Se in maternal diet on regulation of gap junction expression and function in fetal tissues.

In summary, Cx26, Cx32, Cx37 and Cx43 are expressed in several ovarian compartments but the distribution pattern of these connexins differed within fetal ovaries in sheep. The presence of these connexins in fetal ovaries suggests that they may play a differential role in gap junction mediated regulation of cell-to-cell communication, growth and function of fetal ovaries in sheep and possibly in other species. Expression of Cx26 and Cx43 was affected by the stage of follicular development and by maternal diet; HSe in M and R diets decreased Cx26 expression in granulosa layer of antral follicles, and HSe in M diet increased Cx43 expression in granulosa layer of primary and granulosa, and theca layers of antral follicles. Therefore, we postulate that Cx26 and Cx43 are involved in the regulation of folliculogenesis in fetal ovaries. Our results emphasize the importance of maternal diet in the regulation of fetal ovarian growth. The results of this study will help us to understand the role of maternal diet in regulation of gap junction function in fetal tissues.

Although we have demonstrated the effects of maternal diet on fetal ovarian development and suggested a role for connexins in the process, it is currently unclear if dietary supplements would affect oocyte quality and thus the reproductive function of the offspring. Therefore, more research is required to demonstrate if exposure of the fetus to specific environmental factors may affect reproductive function in the offspring [62]. Nevertheless, the knowledge generated in this study will likely contribute to the concept of fetal programming/fetal origin of adult disease.

Acknowledgments

This study was supported by USDA grants 2005-35206-15281 and 2006-38640-16858 to JSC, KAV and DAR, Hatch Project ND01712 and NSF MRI-ARRA grant to ATGB, and by NIH grant P20 RR016741 from the INBRE program of the NCRR to ATGB and LPR. The authors would like to thank Ms. Tammi Neville, Mr. James D. Kirsch, Mr. Kim C. Kraft, Mr. Robert Weigl, Mr. Tim Johnson (deceased), Mr. Terry Skunberg and other members of our laboratory for their technical assistance. We would also like to acknowledge the valuable collaboration and assistance provided by Dr. Bret Taylor and the U.S. Sheep Experimental Station, Dubois, ID.

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Fig. 1.

Representative micrographs of Cx26 immunolocalization in ovine fetal ovaries from group fed maintenance diet with adequate Se. Arrowheads indicate positive staining (dark color) in oocytes of primordial and primary follicles (A, B); small arrows indicate positive staining in oocytes of secondary (S) and antral (An) follicles (A, B, C), large arrows indicate positive staining in granulosa (G) cells of S and An follicles (B, C), and theca (T) cells of An follicle (C), open arrows indicate positive staining in stromal tissues (A, B), and asterisks indicate blood vessels (C, D). Control staining (no primary antibody) did not show any positive staining (E). Bar = 50 μ m.

Fig. 2.

Representative micrographs of Cx32 (A) and Cx37 (B–D) immunolocalization in ovine fetal ovaries from group fed maintenance diet with adequate Se. Green bright fluorescence in (A) indicate positive Cx32 staining in oocyte (O), granulosa (G) and theca (T) layers of antral follicle, but not in stromal tissues (St); red fluorescence indicate cell nuclei; note less staining in St than in G or T layers. Small arrows indicate positive Cx37 staining (dark color) on the border between O and G layer of primordial and primary follicles (B, C). Large arrow indicates positive Cx37 staining on the border between O and G layer of secondary (S) follicle (B). Asterisks indicate positive staining of blood vessels (D). Control staining (no primary antibody) did not show any positive staining (see Fig. 1E). Bar = 50 μ m.

Fig. 3. Representative micrographs of Cx43 immunolocalization (dark color) in primordial (A), primary (B), secondary (C) and antral (D) follicles in ovine fetal ovaries from group fed maintenance diet with adequate Se. Arrows indicate positive staining in flat granulosa (G) layer of primary follicles (A); in G layer of primary and secondary follicle (B, C); in G and theca (T) layers, and on the border between oocyte and G/cumulus cells in antral follicle (D). Control staining (no primary antibody) did not show any positive staining (see Fig. 1E). Bar = 50 μ m.

Fig. 4. Effects of maternal nutrition on expression of Cx26 in granulosa layer of antral follicles (A) and Cx43 in primary (B) and antral follicles (C). ^{a,b}P < 0.01; means $\pm$ SEM with different superscripts differ within follicle type.

Table 1

Number of follicles analyzed for Cx26 and Cx43 expression by using image analysis.

Follicle type	Total number of follicles analyzed (number of sheep per treatment group)		Number of follicles analyzed per nutrition group
	Cx26	Cx43	
Primary	Image analysis not performed	33 (3–4)	6–11
Secondary	126 (5–8)	121 (5–7)	19–43
Antral	108 (5–7)	183 (5–7)	12–67

Table 2

Localization of Cx26, Cx32, Cx37 and Cx43 in ovine fetal ovaries.

Ovarian compartment	Protein
Primordial follicles	Cx26, Cx37 and Cx43
Primary follicles	Cx26, Cx37 and Cx43
Secondary follicles	Cx26, Cx37 and Cx43
Antral follicles	Cx26, Cx32, Cx37 and Cx43
Blood vessels	Cx26 and Cx37
Stromal tissues	Cx26