Connexin43 Is Required for the Maintenance of Multipotency in Skin-Derived Stem Cells

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Expression of the gap junction protein, connexin43 (Cx43), begins early during embryogenesis and is maintained in many different cell types. Several stem cell populations have been shown to express Cx43 and to form functional gap junctions. While it is clear that Cx43 is critical to the function of many organs, whether the same is true for stem cells has not been clearly demonstrated. Recently, stem cells isolated from newborn mouse skin were shown to form oocyte-like cells (OLCs) *in vitro*, hence the present study focussed on the role Cx43 plays in the proliferation and differentiation of these cells. The stem cells express Cx43 and those from knockout mice (Cx43 KO) exhibited significantly reduced cell–cell coupling. Loss of Cx43 reduced the rate of cellular migration [Cx43 KO, 1.57 ± 0.65 radial cell units (RCU); wildtype (WT), 5.57 ± 0.37 RCU] but increased the proliferation rate of the stem cells (Cx43 KO, $29.40\% \pm 2.02\%$; WT, $12.76\% \pm 1.50\%$). The expression of the pluripotency markers OCT4 and Nanog were found to be reduced in the Cx43 KO population, suggesting an inhibition of differentiation potential. To test the differentiation ability, the stem cells were induced to form neuronal cell types *in vitro*. While both the WT and KO cells were able to form GFAP-positive astrocytic cells, only WT stem cells were able to form β III tubulin-positive neurons. Similarly, the ability of the stem cells to form OLCs was ablated by the loss of Cx43. These data reveal a role for Cx43 in maintaining multipotency within the skin-derived stem cell population.

Introduction

GAP JUNCTIONS ARE SPECIALIZED CHANNELS formed in cell membranes by transmembrane proteins termed connexins. They have been shown to allow the movement of molecules smaller than 1 kD between the cytoplasm of two cells [1]. Gap junctions have been shown to play a critical role in many developmental events such as cellular proliferation, apoptosis, differentiation, and organogenesis [2–5]. Connexin43 (Cx43) is considered the most widely expressed connexin and has been shown to play critical roles in many organ systems including folliculogenesis in the ovary, development and function of the heart, and osteoblast differentiation in bone development, among others [5–10]. Along with its role in organ development and function, the increased expression of Cx43 has been shown to decrease the proliferation and invasiveness of many cancer cells [11–13]. Conversely, in other cases the increased expression of Cx43 can lead to an increase in the invasiveness of cancer cells [14]. In other cell types the downregulation of Cx43 has been shown to increase proliferation such as in mouse lung cells, rat osteoblasts, and adrenal cells [15–17]. The ability of Cx43 to have either a positive or negative effect on proliferation

may be explained by the different responses to the protein within different cellular contexts.

Expression of Cx43 begins early during embryogenesis and is maintained in many different cell types [18]. Mouse embryonic stem (ES) cells express this connexin and form functional gap junctions during growth. The importance of Cx43 in the context of ES cells has been demonstrated by knocking Cx43 out or down. When Cx43 is reduced or ablated in mouse ES cells, proliferation is significantly reduced although cellular survival remains unchanged [19,20]. Moreover, the knockout (KO) of Cx43 results in decreased expression of pluripotency markers and increased expression of differentiation markers [20]. This suggests that, in mouse ES cells, Cx43 acts to maintain pluripotency and inhibit cellular differentiation. Mouse ES cells lacking the expression of Cx43 are also unable to form embryoid bodies (EBs) suggesting Cx43 plays a more active role in initiating differentiation.

While it is clear that Cx43 is critical to the function of many organs, the relationship between that essential function and resident stem cells has not been clearly demonstrated. Many somatic tissues contain a local population of stem cells suggested to be responsible for tissue maintenance and repair

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[21], but there is little information on the role that Cx43 plays in those cells. Interestingly, the expression of connexins and functional gap junctions has been shown to be absent in several stem cell populations responsible for epithelium formation such as keratinocytes and corneal epithelium stem cells [22–24]. Also, the reduction of Cx43 function through loss-of-function mutations and genetic knockdown experiments has been found to not inhibit the formation and function of the epidermis [25].

Recently, multipotent stem cells isolated from newborn mice skin have been shown to form oocyte-like cells (OLCs) *in vitro* [26,27]. In the present study, the role (if any) played by Cx43 in the proliferation and differentiation of these cells was investigated. An improved understanding of the role that connexins and gap junctions play in stem cell maintenance and differentiation may help with controlling their traditionally unwieldy differentiation, *in vitro*.

Materials and Methods

Sample collection

All experiments involving animals in the study were conducted according to the Care and Use of Experimental Animals Guidelines of the Canadian Council on Animal Care, and have been approved by the Western University Animal Care and Use Committee. Skin lacking Cx43 was obtained from matings of heterozygous (*Gjal*⁺/*Gjal*[−]) C57BL/6 male and female mice. Fetuses were obtained from pregnant dams at day 17.5–18.5 of gestation after CO₂ anesthesia and cervical dislocation. Fetuses were removed from the uteri and decapitated. A tail snip was collected from each fetus for genotyping. Skin was removed from the back of each fetus and cleaned of fatty tissue, then cultured separately in 1-well of a 24-well plate for 8–10 h in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) and 1× antibiotic/antimycotic (both from Life Technologies) while the fetuses were genotyped.

Genotyping

The polymerase chain reaction (PCR) was applied to proteinase K-digested tail snips to determine the genotypes of the fetal ovary donors. PCR was carried out utilizing two separate reactions that shared a downstream primer (5'-ACTTTTGCCGCCTAGCTATCCC-3') specific for a part of the Cx43 C-terminal cytoplasmic tail-encoding region, which is retained in the null allele [28]. To detect the presence of the WT *Gjal* allele, an upstream primer (5'-CCCCACTCTCACCTATGTCTCC-3') was used in conjunction with the downstream primer, whereas to detect the null allele, an upstream primer located in the neo cassette (5'-GCTTGCCGAATATCATGGTGGA-3') was used with the downstream primer. One microliter of the diluted digestions was used per PCR. Amplifications were carried out using a "touch down" (65°C–58°C) protocol for a total of 40 cycles. PCR products were visualized on a 1% agarose gel containing ethidium bromide (Fisher Scientific, Ottawa, ON, Canada) and documented using a Bio-Rad imaging system and Quantity One software (Bio-Rad, Hercules, CA).

Stem cell isolation

Stem cells were isolated from fetal back skin as previously described [29]. Genotyped skin samples from four to five pups were grouped and placed in Hank's balanced salt solution (HBSS, Life Technologies) and cut into ~1 mm square pieces using dissecting scissors. The samples were then washed 3× using HBSS, and resuspended in 1 mL of 0.05% trypsin for 40 min. at 37°C. Following trypsinization, 1 mL of 0.1% DNase (Life Technologies) was added to the sample and incubated 1 min. at room temperature. Then 9 mL of HBSS was immediately added and the cells were pelleted at 500 g for 5 min. Samples were then washed 1× with HBSS and 2× with DMEM-F12 (Life Technologies) with antibiotics. Following the last wash, the samples were mechanically dissociated in 1 mL of DMEM-F12 by pipetting. The partially dissociated samples were then filtered using a 40 µm cell strainer (BD Biosciences Mississauga, ON, Canada). This was done by adding 9 mL DMEM-F12 to the dissociated cells and running them through the filter. This was followed by 10–15 mL of DMEM-F12. The resulting filtrate was then pelleted by centrifuging for 5 min. at 500 g. Each pellet obtained from four to five pups was then resuspended in 10 mL stem cell medium [DMEM-F12 with 1× B27 serum-free supplement (Life Technologies), 20 ng/mL epidermal growth factor (EGF; Life Technologies), and 40 ng/mL basic fibroblast growth factor 2 (FGF2; Life Technologies)] and plated on a 10 cm dish (Sarstedt, Montreal, QC, Canada). At ~72 h after plating, the skin-derived stem cells grew as suspended spheres, which discriminated them from the rest of the skin cells (attached) in culture. To passage floating cell spheres, medium containing spheres was centrifuged and the pellet was gently dissociated using a large bore pipette. The cells were re-seeded in fresh stem cell medium as above. Cells were passaged every 4–6 days. Sphere sizes were measured using MetaMorph analysis software by collecting images at 24 h using an Olympus (Center Valley, PA) BX-UCB microscope.

Cx43 overexpression

Vectors were constructed as described previously [6]. Briefly, cloned cDNA encoding the full length Cx43 was inserted into the AP2 retroviral vector that contains an internal ribosomal entry site permitting independent translation of the connexin and enhanced green fluorescent protein. Vector lacking connexin cDNA served as negative control. The vector was packaged using 293 GPG packaging cells to produce active virus, which was concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA) according to the manufacturer's protocol. Passage 2 stem cells, pretreated with 2 µg/mL Polybrene (Santa Cruz Biotech, Dallas, TX), were infected for 60 min. with Cx43 WT virus and then the medium was changed for fresh stem cell medium containing 1 µg/mL Polybrene (Santa Cruz Biotech). Stem cells were allowed to recover for 48 h before further experiments were conducted.

RNA isolation and RT-PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's protocol and reverse transcription-polymerase chain reaction

(RT-PCR) was performed as previously described [28]. Real-time PCR was carried out on a CFX96 PCR machine (Bio-Rad) using the SYBR green PCR kit (Life Technologies): 1 μ L of DNase-treated cDNA (from a 20 μ L RT reaction) was added to 5 μ L of SYBR green mix and 0.3 μ M each of forward and reverse primers (final volume 10 μ L). The optimal annealing temperature was determined for each primer pair using a gradient PCR reaction. Product sizes were confirmed on 1% agarose gels. Positive control tissues for connexin expression were as follows: skin was used to test for the transcripts that encode Cx30, 30.3, 31, 31.1, 26, 40, and 45; liver for Cx26 and 32; ovary for Cx43 and 37; brain for Cx47, 29, and 36; eye for Cx57, 50, 46, 36, and 23; developing muscle for Cx39. Primers, expected product sizes, and accession numbers are presented in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd). Primers for the housekeeping gene RNA polymerase II (RpII) were used to perform relative quantification using CFX manager software (Bio-Rad).

Western blotting

For immunoblotting, protein from cells was isolated using radioimmunoprecipitation assay lysis buffer with complete mini protease inhibitors (Roche, Laval, QC, Canada) added fresh prior to use. Thirty micrograms of protein (as determined using a BCA protein assay kit; Pierce, Rockford, IL) were mixed with 5 $\times$ reducing sample buffer, boiled for 5 min, and electrophoresed under reducing conditions on 12% polyacrylamide gels. Protein was transferred using an iBlot (Life Technologies) onto nitrocellulose membranes. Membranes were incubated for 2 h in 5% non-fat dry milk blocking buffer TBST room temperature, followed by an overnight incubation at 4°C in anti-GAPDH antibody (1:5,000; Chemicon, Billerica, MA) or anti-HSC70 (1:10,000; Life Technologies). After a 1 h incubation with anti-mouse IgG (HRP, 1:10,000; Life Technologies) or anti-rat IgG (HRP, 1:10,000; Life Technologies) at room temperature, GAPDH or HSC70 protein was detected, using an ECL assay (GE Healthcare, Baie d'Urfe, QC, Canada) following three washes, using the Bio-Rad VersaDoc imaging system. Blots were incubated in 1 $\times$ strip buffer (re-blot plus mild; Millipore) at room temperature for 15 min and re-blocked 1 h. Antibody incubations were repeated overnight at 4°C with anti-CX43 antibody (1:5,000; Sigma-Aldrich, Oakville, ON, Canada), anti-DAZL antibody (1:500; Sigma-Aldrich), anti-OCT4 (1:300; Santa Cruz Biotech), anti-NANOG (1:500; Santa Cruz Biotech), anti-SYCP3 (1:500; Abcam, Toronto, ON, Canada), or anti-ZP3 (1:800; Santa Cruz Biotech) followed by incubations with anti-rabbit IgG (HRP, 1:10,000; Life Technologies) or anti-goat (HRP, 1:10,000; Life Technologies). All blots were stripped, blocked, and re-probed between antibodies. Expression levels were determined by normalizing the expression to HSC70 and GAPDH housekeeping proteins.

Immunofluorescence

Cells were washed twice with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS for 20 min. They were then washed thrice in PBS with 0.1% Tween 20 (Fisher Scientific, Ottawa, ON, Canada) and incubated for 10 min, and then for 20 min in PBS with 1%

Triton X-100 (Fisher Scientific). Next, cells were blocked for 2 h in PBS with 5% bovine serum albumen (BSA; Roche), and 0.05% Triton X-100 (PBS-B, blocking solution), followed by incubation with primary antibody, either 1:5,000 anti-Cx43 (Sigma-Aldrich), 1:400 anti-GFAP (Sigma-Aldrich), or 1:300 anti- β III tubulin (Sigma-Aldrich) for 2 h at 37°C, or overnight at 4°C. Cells were then washed in blocking solution (PBS with 5% BSA and 0.05% Triton X-100; Fisher Scientific), and incubated with 1:500 phycoerythrin-conjugated goat anti-rabbit IgG (Life Technologies) or 1:500 fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG (Life Technologies) for 1 h at room temperature. This was followed with a blocking solution wash and incubation with Hoechst 33342 (Life Technologies) for 10 min, followed by washing thrice with PBS-B. Cells were mounted using PVA (Sigma-Aldrich) and viewed using an Olympus BX-UCB microscope equipped with MetaMorph analysis software (Universal Imaging Corporation, Downingtown, PA).

Testing for functional gap junctions

The presence of gap junctions in the cultured stem cells was determined by fluorescent dye injection. Lucifer yellow (0.5%; Life Technologies) was injected using a pulled glass pipette by filling the pipette via back loading and injecting using a InjectMan NI2 and FemtoJet (Eppendorf, Mississauga, ON, Canada). Cells were allowed to fill for 90 s at which time the number of cells containing dye was recorded using a Leica DMIRE2 and Volocity 6.3 software. Cells were considered functionally coupled when the dye spread from the injected cell to surrounding cells.

Cell proliferation assay

The Click-iT EdU Imaging Kit (Life Technologies) was used to measure cell proliferation. Cells were cultured with 10 μ M 5-ethynyl-2'-deoxyuridine (EdU) for 6 h then fixed in 4% PFA for 20 min. The fixative was washed off using PBS containing 3% BSA (PBS-BSA) and then the cells were incubated for 20 min with 0.5% Triton X-100, washed once with PBS-BSA, and incubated 30 min with the Click-iT reaction cocktail followed by washing with PBS-BSA. Nuclei were labeled using the Hoechst dye provided in the kit and according to the manufacturer's protocol. The coverslips were mounted onto slides using PVA (Sigma-Aldrich) and documented using a fluorescence photomicroscope (Leica Microsystems, Concord, ON, Canada). The ratio of EdU-positive cells to total cells was determined by counting five fields per coverslip and each experiment was conducted thrice. To test the sphere-forming ability of the stem cell populations, samples were completely dissociated and serially diluted into 96-well plates. The presence of a single cell per well was confirmed and those wells containing no or more than one cell were discarded from analysis. The cells' ability to re-form spheres was assessed following 5 days of culture.

Cellular migration

Stem cells were removed from stem cell medium and placed in DMEM-F12 supplemented with 10% FBS for 24 h on collagen-coated coverslips. The cells were allowed to

attach for 24 h and then images were collected. The images were analyzed by counting the monolayer cells radiating out from the edge of the spheres, radial cell units (RCU), and the data were analyzed using a *t*-test.

Neuronal differentiation

For neuronal differentiation, spheres were centrifuged, mechanically dissociated by pipetting, and resuspended in fresh medium containing B27 and 1% FBS (Life Technologies). Single cells dissociated from spheres were plated onto plates coated with a solution of gelatin (0.1%; Life Technologies) to facilitate cell attachment. From 24 h onward, the neuronal differentiation group was cultured for 7 days in DMEM-F12 (1:1) containing antibiotics, B-27 without serum, and growth factors. Half of the medium was changed every 2 days.

Differentiation into germ cells

The isolated stem cells at passage 2 were pelleted at 500 *g* and resuspended in 500 μ L of PBS. The cells were dissociated to single cells by using vigorous pipetting. The cells were then washed in 9 mL of PBS and counted on a hemocytometer. Cells were plated at 0.6×10^6 cells/well (500 μ L) on a flat bottom 24-well suspension culture plate in differentiation medium that consists of M199 (no antibiotics; Life Technologies) supplemented with 0.05 IU follicle stimulating hormone (Sioux Biochemical, Sioux Center, IA), 0.03 IU luteinizing hormone (Sioux Biochemical), 3 mg/mL BSA, 5 μ L/mL ITS (Life Technologies), 0.23 mM sodium pyruvate (Life Technologies), 1 mg/mL fetuin (Sigma-Aldrich), and 1 ng/mL EGF (Life Technologies). The cells were cultured at 37°C for 12 days, changing half

of the medium every 3 days. Spent medium was centrifuged and the pelleted cells returned to the culture dish.

Apoptosis assay

Isolated stem cells at early passage 2 were analyzed using an Image-iT LIVE Green caspase-3 and -7 Detection Kit (I35106; Life Technologies). Briefly, cells were cultured for 60 min in the presence of $1 \times$ FLICA reagent in stem cell culture medium. Cells were then gently rinsed in fresh stem cell culture medium and incubated for 10 min in the presence of 1μ M Hoechst 33342. Following two washes in $1 \times$ wash buffer cells were then mounted using the provided mount containing fixative. Cells were immediately documented using an Olympus BX-UCB microscope equipped with FITC and DAPI filters and MetaMorph analysis software (Universal Imaging Corporation).

Results

Skin-originating stem cells express Cx43 and form functional gap junctions

The expression of connexins was investigated using real-time RT-PCR. Skin-originating stem cells were found to express transcripts encoding Cx43 and connexins 26, 29, 30, 30.3, 31, 33, 37, 39, 40, 46, and 50 (Fig. 1A). The normalized expression level of transcripts encoding Cx43 was higher than that of the other connexins detected (Fig. 1B). To confirm the presence of Cx43 in gap junctions, its expression was investigated using immunofluorescence. Cx43 was detected in gap junction plaque-like structures between the stem cells (Fig. 1C). The presence of functional gap junctions between the cells was tested using a Lucifer yellow dye transfer assay: the injected dye spread to surrounding cells

FIG. 1. Expression profile of connexin transcripts and functional coupling in mouse skin-derived stem cells. (A) Reverse transcription-polymerase chain reaction (RT-PCR) products of connexin transcripts in the stem cells and in positive control tissues. (B) Primers for the housekeeping gene RNA polymerase II (RpII) were used to perform relative quantification of the various connexin transcripts present in the stem cell population. (C) Connexin43 (Cx43)-based gap junction plaques (green immunostain) are present in the stem cells. (D) The spread of Lucifer yellow from the injected cell (*) to the surrounding cells exhibits functional cell coupling. (E) Western blot confirmed the presence of Cx43 (both native and phosphorylated forms, P0, P1, P2, P3) in the WT stem cells and absence of Cx43 in the knockout (KO) stem cells. (F) The KO stem cells have a significantly lower coupling rate compared with WT stem cells (**P* < 0.05). WT, wild-type. Scale bars = 20 μ m. Color images available online at www.liebertpub.com/scd

showing evidence of functional coupling between the cells (Fig. 1D). The expression of Cx43 was then compared between stem cells isolated from WT and Cx43 KO (*Gja1*^{-/-}) newborn skin using western blots (Fig. 1E). As expected, the KO cells did not express any detectable Cx43 protein (Fig. 1E) and the dye transfer rate was significantly reduced when compared with the WT stem cells (Fig. 1F). These results indicate that Cx43 plays a key role in maintaining metabolic coupling between the stem cells.

Loss of Cx43 alters stem cell isolation metrics

Figure 2A and B depicts the morphology of skin-originating spheres isolated from WT and Cx43 KO mice at passage 2. The spheres generated from the two strains of mice were similar in size ($170.9 \pm 3.16 \mu\text{m}$ WT vs. $173.3 \pm 3.86 \mu\text{m}$ KO) at passage 2 (Fig. 2B). However, there were more spheres generated in the Cx43 KO isolations (6 ± 0.58 vs. 2.67 ± 0.67 spheres per 10 microliters, Fig. 2D). A sphere-forming assay was conducted to determine potential differences in the ability of the stem cells to clonally expand. Serially diluted cells were plated for both Cx43 KO and WT populations and the ability of the single cells to re-form spheres was assessed. A significantly higher percentage of Cx43 KO cells ($61.66\% \pm 4.44\%$) was able to re-form spheres when compared with WT populations ($33.81\% \pm 1.90\%$, Fig. 2E, $P < 0.001$).

Loss of Cx43 alters stem cell migration and proliferation but not apoptosis rate

To determine whether the loss of Cx43 altered the stem cells' ability to migrate, an in vitro migration assay was used. Spheres with similar diameters were selected and plated on gelatin-coated coverslips for 24h. Cells within the spheres migrated outward radially following adhesion (Fig. 3D–F). As shown in Figure 3, loss of Cx43 significantly attenuated the ability of the stem cells to migrate out of the spheres when compared with WT and Cx43-overexpressing controls (Fig. 3C: Cx43 KO, 1.57 ± 0.65 RCU; WT, 5.57 ± 0.37 RCU; Cx43 overexpressing, 6.714 ± 0.52 RCU). Furthermore, loss of Cx43 resulted in a significantly higher rate of proliferation relative to WT or Cx43-overexpressing stem cells, as shown by comparing EDU incorporation rates (Fig. 3A: KO, $29.40\% \pm 2.02\%$; WT, $12.76\% \pm 1.50\%$; Cx43 overexpressing, $19.16\% \pm 0.85\%$). The absence of Cx43 did not effect the rate of apoptosis as determined by caspase-3 and -7 activation (Fig. 3B).

Loss of Cx43 alters the expression of OCT4 and Nanog

To test the ability of the stem cells to differentiate following Cx43 loss, the expression of transcripts encoding the pluripotent markers OCT4 and Nanog were compared using real-time RT-PCR. As shown in Figure 4A, the expression of

FIG. 2. Isolation and growth characteristics of stem cells isolated from WT and Cx43 KO mouse skin. (A, B) A similar morphology was seen at passage 2 of skin-derived stem cells isolated from WT and Cx43 KO mice. (C) There was no significant difference in sphere volume between cells of the two genotypes. (D) The number of spheres isolated was significantly higher in the Cx43 KO stem cell cultures ($P < 0.05$). (E) The ability of the cells to clonally expand, quantified as sphere-forming units (SFUs), was higher in the Cx43 KO cultures ($P < 0.01$). Scale bars = $50 \mu\text{m}$.

FIG. 3. Proliferation and cellular migration are affected by Cx43 expression level but apoptosis rate is not. (A) The proliferation rate of Cx43 KO stem cells was significantly higher than that of both WT and Cx43-overexpressing (Inc Cx43) cells. (B) The rate of activated caspase-3 and -7 was not significantly different between the Cx43 WT and KO stem cells. (C–F) Conversely, both WT and Cx43-overexpressing cells displayed greater propensity for cellular migration (indicated by the number of cell layers radiating outward from the edge of the colony) when compared with KO cells. Results were analyzed using a one-way ANOVA with a Tukey's post-test (A, C; $P < 0.05$) and a t -test (B). Scale bars = 50 μ m. Different letters above bars indicate significant differences.

FIG. 4. The expression of OCT4 and Nanog is affected by Cx43 levels. (A) The transcript encoding OCT4 was significantly higher in WT stem cells when compared to Cx43 KO stem cells (* $P < 0.02$). (B) Similarly, OCT4 itself was higher in WT stem cells (* $P < 0.05$) when normalized to HSC70 expression. Conversely, the transcript encoding Nanog was found to not be significantly different between the WT and KO stem cells (C). However, Nanog itself was significantly higher in the Cx43 WT stem cells when compared with the Cx43 KOs (D). (* $P = 0.05$).

Oct4 in the Cx43 KO cells was significantly less than that seen in the WT cells. Conversely, the expression of *Nanog* was similar between the WT and KO cells (Fig. 4C). When compared at the protein level, the expression of OCT4 in the KO cells was also significantly lower than that in the WT cells (Fig. 4B, $13.67\% \pm 2.47\%$ vs. $29.72\% \pm 3.96\%$). Similarly, the expression of Nanog protein was significantly lower in the KO cells when compared with the WT (Fig. 4D, $0.82\% \pm 0.07\%$ vs. $1.263\% \pm 0.08\%$).

Loss of Cx43 alters the neuronal potential of the stem cells

Dissociated stem cell spheres were induced to differentiate for 7 days to study their ability to form neuronal cell types in vitro. As depicted in Figure 5A, both genotypes were able to form astrocytes (GFAP-positive cells), however, Cx43 KO cells were unable to form β III tubulin-positive neurons following differentiation. As shown in Figure 5B, the expression of transcripts encoding nestin, a neural progenitor marker, decreased in both KO and WT cells following induced differentiation. Conversely, as seen in Figure 5C, the expression of β III tubulin-encoding transcripts only increased in the WT cells, remaining unchanged in the Cx43 KO cells, supporting a failure of neuron formation.

Loss of Cx43 alters the germ cell potential of stem cells

During germ cell differentiation in the stem cell cultures, morphologically distinct, suspended round cells (OLCs) form between days 4 and 8. These cells express OCT4 as

shown in the representative images in Figure 6A. The ratio of OCT4-positive cells to OCT4-negative cells was higher in the WT differentiations than those of the Cx43 KO cells (Fig. 6B). Expression of the early stage germ cell marker DAZL was also compared between the WT and Cx43 KO differentiations at day 8 (Fig. 6C) revealing that the level of DAZL expression was lower in the KO cultures. These data support an inhibition of germ cell formation in the absence of Cx43. The expression of pluripotent markers was also tested at late stages of differentiation (days 12–14). The expression of OCT4 was higher both before and after induced differentiation in the Cx43 WT cultures (Fig. 7A) but expression of the pluripotent marker NANOG remained unchanged in both groups following differentiation (Fig. 7B). Conversely, the expression of DAZL was higher in WT differentiations than those lacking Cx43. Meiosis-specific SYCP3 and the oocyte-specific protein ZP3 were both higher in the WT differentiations. The growth of OLCs makes them easy to identify by day 12–14 of induced differentiation. While large OCT4-positive OLCs were present in the WT differentiations, in those lacking Cx43 no OLCs could be recovered (Fig. 7C).

Discussion

Connexins play important roles in many different cell types and are involved in cellular proliferation, homeostasis, and differentiation. Within the developing ovarian follicle of the mouse, connexins 37 and 43 have been shown to play differing but critical roles [5,30] with Cx43 being the main contributor to gap junctions formed between granulosa cells, the somatic cells surrounding and nurturing the growing oocyte. Recently, stem cells isolated from mouse skin have

FIG. 5. (A) GFAP-positive astrocytes were detected following differentiation of both WT (*top right panel*) and Cx43 KO (*bottom right panel*) stem cells, but only WT stem cells were able to form β III tubulin-positive neurons (*top left panel*). (B) The expression of the neuronal progenitor marker nestin decreased in cultures of both genotypes following differentiation. (C) The transcript of β III tubulin increased with differentiation in the WT cultures but remained unchanged in the Cx43 KO cultures. Scale bars = 20 μ m. Data in (B, C) were analyzed using an ANOVA with Tukey's post-test. Bars with different letters are significantly ($P < 0.05$) different. Color images available online at www.liebertpub.com/scd

FIG. 6. Expression of OCT4 and DAZL during induced early stage oocyte-like cell (OLC) differentiation. (**A**, **B**) The frequency of OCT4-positive cells was significantly higher in the WT cultures (*t*-test, $*P < 0.05$). (**C**) DAZL expression was higher in the WT cultures (*t*-test, $P < 0.05$). Scale bars = 50 μ m. Color images available online at www.liebertpub.com/scd

been shown to form OLCs in vitro [27]. The present study was undertaken to determine what role, if any, Cx43 plays in the process of OLC formation and its possible importance in the stem cells to regulate proliferation, maintenance of multipotency, and differentiation. Current knowledge in-

volving the roles of connexins in stem cells is largely limited to ES cells. The transcripts encoding several connexins including Cx26, Cx30.3, Cx31, Cx32, Cx37, Cx43, and Cx45 are expressed in ES cells although only Cx31, Cx43, and Cx45 are detectable as proteins [19]. As shown in

FIG. 7. Loss of Cx43 impairs the ability of skin-derived stem cells to form OLCs in vitro. (**A**) OCT4 expression was higher in both undifferentiated (Un) and differentiated (Diff) WT stem cells compared with Cx43 KO stem cells. (**B**) The expression of Nanog remained similar in stem cells of the two genotypes, whereas the expression of DAZL, SYCP3, and ZP3 was higher in WT stem cells. (**C**) OLCs were only detected in the WT stem cell cultures. The expression of OCT4 is revealed by green immunofluorescence. Scale bars = 50 μ m.

Figure 1A, the skin-derived stem cells express the transcripts of many connexins. The expression of transcripts encoding Cx43 were found to be relatively higher than the other expressed connexin transcripts suggesting it is the main connexin contributing to cell coupling (Fig. 1B). Moreover, the KO of Cx43 resulted in a significant decrease in the coupling rate (Fig. 1F), further supporting its role in functionally coupling the stem cells. In addition, the stem cells were found to have Cx43-based gap junctional plaques throughout the population (Fig. 1C) and to be functionally coupled as revealed through dye injection experiments (Fig. 1D). These data support Cx43 contributing to coupling within the stem cell population. However, the total number of connexin family members present at the protein level in the stem cells, and their importance for proliferation and differentiation, remains to be elucidated.

Upon isolation of the skin stem cell populations expressing or lacking Cx43, it was very apparent that the spheres in the Cx43 KO population were more prominent. This could be caused by a greater proportion of cells responding to EGF and FGF2 in the Cx43-deficient population, thus producing more spheres, or an increased aggregation within that population, leading to fewer but larger spheres. To answer this question, spheres were randomly collected and measured for volume in addition to the number of spheres being determined (Fig. 2). The size of the spheres in the Cx43 KO and WT populations did not significantly differ (Fig. 2A–C) but the number of spheres in the KO group was significantly higher when compared with the WT population (Fig. 2A, B, D). This suggests that, in the initial starting Cx43-deficient cell population, there are more cells responsive to the culture conditions. Several studies have looked at the effect of reducing or eliminating Cx43 from ES cells. The knock-down of Cx43 resulted in a decrease in proliferation rate, reduction of pluripotent markers, and an increase in differentiation markers [19,20]. This contrasts with our results regarding proliferation of somatic skin stem cells where proliferation increased when Cx43 was ablated in the population (Fig. 3A). This finding is not particularly surprising as the loss of gap junctional coupling resulting in higher proliferation rate has been found in many cell types including many cancer cells [31,32]. To determine whether the proliferation differences seen were a result of a change in the apoptotic rate, the activation of caspase-3 and -7 was examined. No significant differences in the amount of activated caspases were found between the Cx43 WT and KO stem cells (Fig. 3B). Another metric often examined in stem cells and cancer cells is the ability of the population to migrate: the potential of stem cells to migrate can improve their therapeutic potential while in cancer cells the invasiveness is linked to poorer patient outcomes. In our study, overexpression of Cx43 did not have a significant impact on migration, however, migration was significantly inhibited in the Cx43-deficient stem cells (Fig. 3C–F). Similarly, a reduction in Cx43 has been shown to negatively impact endothelial progenitor cells' ability to migrate, thereby reducing their therapeutic ability [33]. It is apparent from the data presented here that stem cells isolated from Cx43-deficient mice are present in higher numbers and proliferate faster, but are inhibited in their ability to migrate.

The absence of Cx43 in our skin-derived stem cells resulted in decreased expression of OCT4 (Fig. 4A, B) and Nanog (Fig. 4D), consistent with the loss of pluripotency markers previously seen in Cx43 KO ES cells [20]. This decrease in OCT4 expression led us to propose that the ablation of Cx43 affects the cells' ability to maintain multipotency and thus their potential differentiation pathways. To test this, we induced the stem cells to form neuronal cell types. Following differentiation, the expression of the neuronal progenitor marker, nestin, was reduced in both the WT and Cx43 KO populations (Fig. 5B) and the cells stained positive for the astrocyte marker GFAP, indicating they maintained the ability to differentiate. Conversely, the expression of the neuron marker β III tubulin only increased in the WT population and only those cultures formed cells with a neuronal phenotype; the Cx43-deficient population did not form neurons, indicating a crucial role for Cx43 in neuronal differentiation (Fig. 5A).

OLCs represent another differentiation endpoint that stem cell populations, including skin-derived stem cells, have shown the ability to achieve in vitro [27,34–38]. Previous research with mice lacking Cx43 has highlighted the critical role that this connexin plays in oocyte development [5,30,39,40]. Cx43 has been shown to be required for the formation of functional oocytes, but this requirement appears to be restricted to the somatic component of the follicle: if Cx43 is absent only in the oocyte but present in the supporting granulosa cells, follicles progress to the antral stage and the oocytes are able to be matured and fertilized [30]. This indicates that the oocyte does not need to express Cx43 to become fertilizable but its expression is required by the surrounding support cells. During OLC formation from mouse skin-derived stem cells, the expression of some granulosa cell markers such as FSHR, STAR, and CYP19A1 has been detected [27]. Conversely, the expression of P450 C17, which is also involved in the production of estradiol, was not detectable, nor was there estradiol in the spent medium [27]. Thus, the presence of functional secondary support cells within the OLC-forming cultures remains to be clearly demonstrated. In the present study, we tested the effect of Cx43 ablation on the ability of skin-derived stem cells to form OLCs. We hypothesized that if the OLCs are developing independently of the surrounding cells, the ablation of Cx43 would not negatively impact their formation. In comparison with WT cultures, expression of OCT4 was reduced in the Cx43 KO differentiations at both early and late stages (Figs. 6A, B and 7A) as were markers of late stage differentiation including DAZL, SYCP3, and ZP3 (Fig. 7B). The expression of Nanog was found to be comparable between the two genotypes (Fig. 7B), a surprising finding given that Nanog is a germ cell-specific homeobox protein that is downregulated following the initiation of meiosis and expression of SYCP3 [40]. Therefore, with fewer pluripotent cells present and more cells entering meiosis in the WT cultures, one would expect less Nanog in the Cx43-deficient cultures. An explanation of this finding remains elusive. OLCs can be readily collected following 12–14 days of differentiation culture, however, OLCs did not form in cultures lacking Cx43 (Fig. 7C). These data suggest that the ability to form OLCs is hindered in the Cx43-deficient cultures due to failure to maintain a pluripotent cell population.

The presence of Cx43 appears critical for maintaining an undifferentiated pluripotent population of stem cells. Upon Cx43 loss, the stem cells change identity, through loss of pluripotent marker expression, and are unable to form certain cell types in vitro. This is consistent with what is seen in ES cell populations, where a decrease of Cx43 results in an inability to form embryoid bodies [20]. Cx43 is involved in ES cell proliferation and differentiation although the specific roles remain to be elucidated. Taken together, the present and previous studies indicate that Cx43 can play an important role in somatic stem cell proliferation and is required in at least some cases for maintaining a pluripotent, undifferentiated stem cell population.

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Author Disclosure Statement

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References

- Kumar NM and NB Gilula. (1996). The gap junction communication channel. *Cell* 84:381–388.
- Vinken M, T Vanhaecke, P Papeleu, S Snykers, T Henskens and V Rogiers. (2006). Connexins and their channels in cell growth and cell death. *Cell Signal* 18:592–600.
- Parekkadan B, Y Berdichevsky, D Irimia, A Leeder, G Yarmush, M Toner, JB Levine and ML Yarmush. (2008). Cell-cell interaction modulates neuroectodermal specification of embryonic stem cells. *Neurosci Lett* 438:190–195.
- Gangoso E, P Ezan, JC Valle-Casuso, S Herrero-Gonzalez, A Koulakoff, JM Medina, C Giaume and A Tabernero. (2012). Reduced connexin43 expression correlates with c-Src activation, proliferation, and glucose uptake in reactive astrocytes after an excitotoxic insult. *Glia* 60:2040–2049.
- Ackert CL, JE Gittens, MJ O'Brien, JJ Eppig and GM Kidder. (2001). Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. *Dev Biol* 233:258–270.
- Dyce PW, RP Norris, PD Lampe and GM Kidder. (2012). Phosphorylation of serine residues in the C-terminal cytoplasmic tail of connexin43 regulates proliferation of ovarian granulosa cells. *J Membr Biol* 245:291–301.
- Gittens JE, AA Mhawi, D Lidington, Y Ouellette and GM Kidder. (2003). Functional analysis of gap junctions in ovarian granulosa cells: distinct role for connexin43 in early stages of folliculogenesis. *Am J Physiol Cell Physiol* 284:C880–C887.
- Rhee DY, XQ Zhao, RJ Francis, GY Huang, JD Mably and CW Lo. (2009). Connexin 43 regulates epicardial cell polarity and migration in coronary vascular development. *Development* 136:3185–3193.
- Chaible LM, DS Sanches, B Cogliati, G Mennecier and ML Dagli. (2011). Delayed osteoblastic differentiation and bone development in Cx43 knockout mice. *Toxicol Pathol* 39:1046–1055.
- Loiselle AE, EM Paul, GS Lewis and HJ Donahue. (2013). Osteoblast and osteocyte-specific loss of connexin43 results in delayed bone formation and healing during murine fracture healing. *J Orthop Res* 31:147–154.
- Ogawa K, P Pitchakarn, S Suzuki, T Chewonarin, M Tang, S Takahashi, A Naiki-Ito, S Sato, M Asamoto and T Shirai. (2012). Silencing of connexin 43 suppresses invasion, migration and lung metastasis of rat hepatocellular carcinoma cells. *Cancer Sci* 103:860–867.
- Liu CL, YS Huang, M Hosokawa, K Miyashita and ML Hu. (2009). Inhibition of proliferation of a hepatoma cell line by fucoxanthin in relation to cell cycle arrest and enhanced gap junctional intercellular communication. *Chem Biol Interact* 182:165–172.
- Sirnes S, J Bruun, M Kolberg, A Kjenseth, GE Lind, A Svindland, A Brech, A Nesbakken, RA Lothe, E Leithe and E Rivedal. (2012). Connexin43 acts as a colorectal cancer tumor suppressor and predicts disease outcome. *Int J Cancer* 131:570–581.
- Bates DC, WC Sin, Q Aftab and CC Naus. (2007). Connexin43 enhances glioma invasion by a mechanism involving the carboxy terminus. *Glia* 55:1554–1564.
- Avanzo JL, M Mesnil, FJ Hernandez-Blazquez, I Mackowiak, CM Mori, TC da Silva, SC Oloris, AP Garate, SM Massironi, H Yamasaki and ML Dagli. (2004). Increased susceptibility to urethane-induced lung tumors in mice with decreased expression of connexin43. *Carcinogenesis* 25:1973–1982.
- Gramsch B, HD Gabriel, M Wiemann, R Grummer, E Winterhager, D Bingmann and K Schirmacher. (2001). Enhancement of connexin 43 expression increases proliferation and differentiation of an osteoblast-like cell line. *Exp Cell Res* 264:397–407.
- Wynn J, U Shah and SA Murray. (2002). Redistribution of connexin 43 by cAMP: a mechanism for growth control in adrenal cells. *Endocr Res* 28:663–668.
- Levin M. (2002). Isolation and community: a review of the role of gap-junctional communication in embryonic patterning. *J Membr Biol* 185:177–192.
- Worsdorfer P, S Maxeiner, C Markopoulos, G Kirfel, V Wulf, T Auth, S Urschel, J von Maltzahn and K Willecke. (2008). Connexin expression and functional analysis of gap junctional communication in mouse embryonic stem cells. *Stem Cells* 26:431–439.
- Todorova MG, B Soria and I Quesada. (2008). Gap junctional intercellular communication is required to maintain embryonic stem cells in a non-differentiated and proliferative state. *J Cell Physiol* 214:354–362.
- Rock JR and BL Hogan. (2011). Epithelial progenitor cells in lung development, maintenance, repair, and disease. *Annu Rev Cell Dev Biol* 27:493–512.
- Matic M and M Simon. (2003). Label-retaining cells (presumptive stem cells) of mice vibrissae do not express gap junction protein connexin 43. *J Invest Dermatol Symp Proc* 8:91–95.
- Priya CG, P Arpitha, S Vaishali, NV Prajna, K Usha, K Sheetal and V Muthukkaruppan. (2011). Adult human buccal epithelial stem cells: identification, ex vivo expansion, and transplantation for corneal surface reconstruction. *Eye (Lond)* 25:1641–1649.
- Matic M, IN Petrov, S Chen, C Wang, SD Dimitrijevic and JM Wolosin. (1997). Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity. *Differentiation* 61:251–260.
- Langlois S, AC Maher, JL Manias, Q Shao, GM Kidder and DW Laird. (2007). Connexin levels regulate keratinocyte differentiation in the epidermis. *J Biol Chem* 282:30171–30180.

26. Toma JG, M Akhavan, KJ Fernandes, F Barnabe-Heider, A Sadikot, DR Kaplan and FD Miller. (2001). Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 3:778–784.
27. Dyce PW, J Liu, C Tayade, GM Kidder, DH Betts and J Li. (2011). In vitro and in vivo germ line potential of stem cells derived from newborn mouse skin. *PLoS One* 6: e20339.
28. Reaume A, PA De Sousa, S Kulkarni, BL Langille, D Zhu, TC Davies, SC Juneja, GM Kidder and J Rossant. (1995). Cardiac malformation in neonatal mice lacking connexin43. *Science* 267:1831–1834.
29. Dyce PW, H Zhu, J Craig and J Li. (2004). Stem cells with multilineage potential derived from porcine skin. *Biochem Biophys Res Commun* 316:651–658.
30. Gittens JE and GM Kidder. (2005). Differential contributions of connexin37 and connexin43 to oogenesis revealed in chimeric reaggregated mouse ovaries. *J Cell Sci* 118: 5071–5078.
31. King TJ, LH Fukushima, TA Donlon, AD Hieber, KA Shimabukuro and JS Bertram. (2000). Correlation between growth control, neoplastic potential and endogenous connexin43 expression in HeLa cell lines: implications for tumor progression. *Carcinogenesis* 21:311–315.
32. Vine AL and JS Bertram. (2002). Cancer chemoprevention by connexins. *Cancer Metastasis Rev* 21:199–216.
33. Wang HH, CH Su, YJ Wu, JY Li, YM Tseng, YC Lin, CL Hsieh, CH Tsai and HI Yeh. (2013). Reduction of connexin43 in human endothelial progenitor cells impairs the angiogenic potential. *Angiogenesis* 16:553–560.
34. Dyce PW, L Wen and J Li. (2006). In vitro germline potential of stem cells derived from fetal porcine skin. *Nat Cell Biol* 8:384–390.
35. Danner S, J Kajahn, C Geismann, E Klink and C Kruse. (2007). Derivation of oocyte-like cells from a clonal pancreatic stem cell line. *Mol Hum Reprod* 13:11–20.
36. Wang L, J Cao, P Ji, D Zhang, L Ma, M Dym, Z Yu and L Feng. (2012). Oocyte-like cells induced from mouse spermatogonial stem cells. *Cell Biosci* 2:27.
37. Ma Z, R Liu, X Wang, M Huang, Q Gao, Y Lu and C Liu. (2013). Spontaneous germline potential of human hepatic cell line in vitro. *Mol Hum Reprod* 19:216–226.
38. Hubner K, G Fuhrmann, LK Christenson, J Kehler, R Reinbold, R De La Fuente, J Wood, JF Strauss 3rd, M Boiani and HR Scholer. (2003). Derivation of oocytes from mouse embryonic stem cells. *Science* 300:1251–1256.
39. Juneja SC, KJ Barr, GC Enders and GM Kidder. (1999). Defects in the germ line and gonads of mice lacking connexin43. *Biol Reprod* 60:1263–1270.
40. Yamaguchi S, H Kimura, M Tada, N Nakatsuji and T Tada. (2005). Nanog expression in mouse germ cell development. *Gene Expr Patterns* 5:639–646.

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