Biochemistry and Biophysics Reports 24 (2020) 100828

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

The transcription factors SF-1 and SOX8 cooperate to upregulate *Cx43* expression in mouse TM4 sertoli cells

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ARTICLE INFO

Keywords:
Cell-to-cell communication
CX43
Sry-related HMG box
SF-1
Sertoli cells

ABSTRACT

Gap junctions made by connexins within the adult testis are essential for communication between Sertoli cells and for spermatogenesis. Sertoli cells play an important role in supporting germ cells differentiation and maturation into spermatozoa. Connexin43 (Cx43) is the most abundant and important connexin of the testis. We have shown previously that the expression of *Cx43* is being regulated by SOX and AP-1 transcription factors in Sertoli cells. However, additional regulatory elements being able to recruit orphan nuclear receptors may be involved. Since SOX and SF-1 transcription factors have been shown to cooperate to regulate gene expression in Sertoli cells, we wondered if such mechanism could be involved in the activation of *Cx43* expression. Thus, the activity of the *Cx43* promoter was measured by co-transfections of luciferase reporter plasmid constructs with different expression vectors for transcription factors in the TM4 Sertoli cell line. The recruitment of SF-1 to the proximal region of the *Cx43* promoter was evaluated by chromatin immunoprecipitation. Our results indicate that SOX8 and SF-1, as well as SOX9 and Nur77, cooperate to activate the expression of *Cx43* and that SF-1 is being recruited to the −132 to −26 bp region of the *Cx43* promoter. These results allow us to have a better understanding of the mechanisms regulating *Cx43* expression and could explain some disturbances in communication between Sertoli cells responsible for impaired fertility.

1. Introduction

During spermatogenesis, Sertoli cells provide nutrients and protection to germ cells for division, differentiation and maturation. In the testis, intercellular communication is important for the regulation of proliferation and differentiation of somatic and germ cells [1]. In addition to releasing various endocrine and paracrine agents, Sertoli cells can communicate with each other through gap junctions. These junctions allow the exchange of signaling molecules (<1 kDa) such as cyclic AMP, inositol phosphates, ATP, amino acids, micro-RNAs and several coenzymes between Sertoli cells and other cells of the seminiferous tubules [2–5]. It is well known that interactions between Sertoli cells are essential for normal spermatogenesis [6]. Gap junctions between neighbouring cells are formed by the interaction of connexon units, each composed of six connexin proteins. Of these subunits, connexin43 (Cx43, Gja1) is the most abundant and important connexin in the testis. Its absence causes infertility, disruption of spermatogenesis, loss of the blood-testis barrier and reduced size of the testes [6,7]. In addition, the replacement of Cx43 by other related connexins (Cx32, Cx40 or Cx26) does not compensate for its important role in male reproduction [7].

In the testis, the transcription factors SOX9 and SOX8 are expressed during postnatal development [8,9]. In addition, the expression of SOX8 in Sertoli cells is important for the maintenance of spermatogenesis, male fertility and contributes to sex determination [10–12]. The activities of SOX transcription factors are influenced by the formation of dimers [9,13,14] and by post-translational modifications such as phosphorylation, sumoylation and acetylation [15–17]. It is known that SOX factors, including SOX8 and SOX9, can interact with the orphan nuclear receptor SF-1 (NR5A1) [9,18]. In Sertoli cell precursors, SOX9 plays a critical role in male sex determination by regulating the expression of the anti-Müllerian hormone (AMH) by cooperation with SF-1 [18–20]. In addition, the nuclear receptor SF-1 is important for sex development, steroidogenesis and the expression of *Sox9* in Sertoli cells [21].

Interestingly, the *Cx43* promoter harbours several highly conserved regulatory elements for SOX and SF-1 transcription factors and these are known to cooperate to regulate gene expression important for Sertoli

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https://doi.org/10.1016/j.bbrep.2020.100828
Received 28 June 2020; Received in revised form 23 September 2020; Accepted 27 September 2020

cells [9,20]. Furthermore, we have shown that SOX8/SOX9 can cooperate with cJUN to activate the expression of *Cx43* in Sertoli cell lines [22]. Hence, since SOX family members can cooperate with SF-1 to regulate *Amh* expression, we investigated if such cooperation was also involved in the upregulation of *Cx43* expression within Sertoli cells. Indeed, we report that SOX8 and SF-1 synergistically activate the mouse *Cx43* promoter in mouse TM4 Sertoli cells.

2. Materials and methods

2.1. Plasmids

Luciferase reporters containing the mouse *Cx43* promoter constructs (−1697, −1042, −469, −132 and −26 bp) have been described previously [23]. The rat Nur77 (NR4A1), Nurr1 (NR4A2) and NOR1 (NR4A3) expression vectors [24] were kindly provided by Dr. Jacques Drouin (Laboratoire de Génétique Moléculaire, Institut de Recherches Cliniques de Montréal, Montréal, QC). The mouse SF-1 (NR5A1) expression vector has been described previously [25]. The human LRH-1 (NR5A2) expression vector [26] was provided by Dr. Luc Bélanger (Centre de recherche en cancérologie, Centre de recherche du CHUQ, Université Laval, Québec, Canada). The mouse SOX8 and SOX9 expression vectors have been described previously [27,28] and were generously provided by Dr. Michael Wegner (Institut für Biochemie, Universität Erlangen, Germany) and Dr. James Wells (Cincinnati Children's Hospital Research Foundation, Cincinnati, OH), respectively. The mouse SOX4 expression vector was purchased from OriGene (Cat #: MR207005, OriGene Technologies, Inc., Rockville, MD). The SOX8 deletion construct (DN-SOX8), lacking amino acids 176–464 (Δ176–464), has been described previously [22].

2.2. Cell culture and transfections

The mouse TM4 Sertoli cell line [29] was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured as previously reported [22]. Cells were transfected using polyethylenimine according to the previously described method [23]. Forty-eight hours after transfection, cells were lysed and luciferase activities were measured using a Varioskan luminometer (Thermo Scientific, Waltham, MA, USA).

2.3. Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as described previously [22] with minor modifications. Protein-DNA complexes were incubated using 1 μl of normal rabbit antibody (Cat.: 2729, Cell Signaling), 10 μl of Histone H3 antibody (Cat.: 4620, Cell Signaling Technology, Danvers, MA, USA), 10 μl of cJUN antibody (Cat.: 9165, Cell Signaling Technology, Danvers, MA, USA) or 10 μl of SF-1 antibody (Cat.: 12800S, Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C with rotation, followed by immunoprecipitation using ChIP-Grade Protein G Magnetic Beads. After beads' washings, reversal of cross-linkings and DNA purification using spin columns, quantitative PCRs were performed using previously described primers targeting the −731 to −547, −554 to −447, −313 to −179 and −153 to +46 bp regions of the mouse *Cx43* promoter [23]. Amplification of a region of the second intron of *Rpl30* (Cat.: 7015, Cell Signaling Technology, Danvers, MA, USA) served as a negative control for cJUN and SF-1 DNA recruitments. ChIP results were confirmed by three separate experiments.

2.4. Analyses of publicly available datasets

Expression levels for *Sox* members, *Sf-1, Nur77* and *Cx43* from purified mouse Sertoli cells according to postnatal development were determined using publicly available RNA libraries (NCBI SRA: PRJNA255976) [30]. Raw reads in FASTQ format were aligned to reference mouse genome (mm10) using RNA-STAR [31]. Gene expression levels and comparisons between Sertoli cells developmental stages were performed using DESeq2 [32] from the Galaxy web-based platform (https://usegalaxy.org/) and presented as percent fold change expressions compared to post-natal day 5 (P5).

2.5. Statistical analyses

Statistical analyses were performed on fold activation results using a one-way or two-way analysis of variance (ANOVA) followed by the appropriate multiple comparison test as indicated in figure legends. For all statistical analyses, $P < 0.05$ was considered significant. All statistical analyses were completed using the GraphPad Prism 8.3.1 software package (GraphPad Software Inc, La Jolla, CA).

3. Results

3.1. Functional cooperation between SOX8 and SF-1 activates the mouse Cx43 promoter

Several potential SOX8 and SF-1 DNA regulatory elements are conserved between species and present within the *Cx43* promoter (Fig. 1A), suggesting that these transcription factors may cooperate to activate *Cx43* expression. To determine if SOX transcription factors can cooperate with members of the NR5A family to activate *Cx43* expression, we co-transfected a −1697 to +72 bp (hereby referenced as full-length) mouse *Cx43* promoter/luciferase reporter construct along with the expression plasmids encoding the orphan nuclear receptors SF-1 or LRH-1 in combination with plasmids encoding SOX4, SOX8 or SOX9 in TM4 Sertoli cells (Fig. 1B–D). Although the combination of SOX9 and SF-1 activated the *Cx43* promoter (Fig. 1C), only the combinations between SOX8 and SF-1 or LRH-1 were able to synergistically increase *Cx43* promoter activity (Fig. 1D). Interestingly, use of a dominant negative expression construct of SOX8, lacking amino acids 176–464, resulted in loss of synergistic activation with SF-1 of the *Cx43* promoter in TM4 cells (Fig. 1E).

3.2. Location of the promoter region involved in the cooperation between SOX8 and SF-1 to activate Cx43 expression

To delineate the promoter region involved in SOX8 and SF-1 dependent activation, 5′ deletions of the mouse *Cx43* promoter were generated and tested for SOX8 and/or SF-1 responsiveness in TM4 Sertoli cells. As indicated in Fig. 2, a significant activation of the *Cx43* promoter was observed following co-transfection of SOX8 and SF-1 along with 5′ deletion constructs from −1697 to −132 bp. However, deletion from −132 to −26 bp resulted in a complete loss of *Cx43* promoter activation by SOX8 and SF-1. These results suggest that DNA regulatory elements for SOX8 and SF-1 dependent activation may be located between −132 and −26 bp of the *Cx43* promoter.

3.3. The cooperation between SOX8 and SF-1 involves recruitment of SF-1 to the proximal region of the Cx43 promoter

To determine if the proximal region of the *Cx43* promoter is involved in the recruitment of SF-1, we performed chromatin immunoprecipitation assays. Following immunoprecipitations using IgG, H3, cJUN or SF-1 antibodies, protein-DNA complexes were washed, DNA purified and subjected to quantitative PCR assays. For every DNA region investigated, a positive amplification for protein-DNA enrichment using histone H3 antibody served as a positive control, whereas amplification for protein-DNA enrichment using IgG served as a negative control (Fig. 3). Interestingly, only the amplification of the −153 to +46 bp region of the *Cx43* promoter showed a protein-DNA enrichment for SF-1 (Fig. 3D) and confirmed that SF-1 was being recruited to this region. In addition, two regulatory elements for SF-1 at −94 and −76 bp are highly conserved in

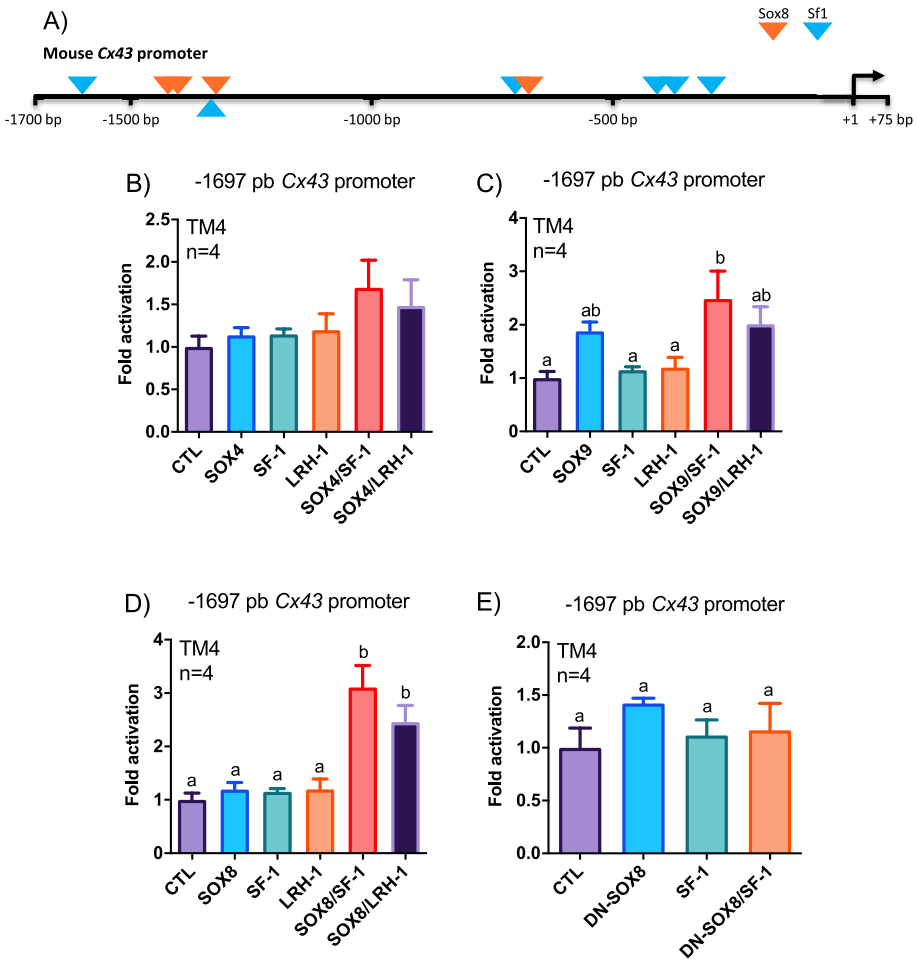

Fig. 1. Regulation of the mouse *Cx43* promoter by SOX, SF-1 and/or LRH-1 transcription factors. Regulatory elements for SOX8 and SF-1 within the *Cx43* promoter region have been identified using the JASPAR CORE 2018 database (A). TM4 Sertoli cells were co-transfected with combinations of the full-length *Cx43* promoter (−1697 bp to +72 bp) luciferase reporter along with expression vectors for: SOX4, SF-1 and LRH-1 (B); SOX9, SF-1 and LRH-1 (C); SOX8, SF-1 and LRH-1 (D); dominant-negative (DN) version of SOX8 and SF-1 (E). CTL corresponds to transfection of the full length *Cx43* promoter-reporter plasmid with an empty expression vector. The number of experiments, each performed in triplicate, is indicated. Results are shown as fold activation over control (±SEM). Statistical analyses were performed using a two-way ANOVA followed by a Tukey multiple comparisons test (different letters: $P < 0.05$).

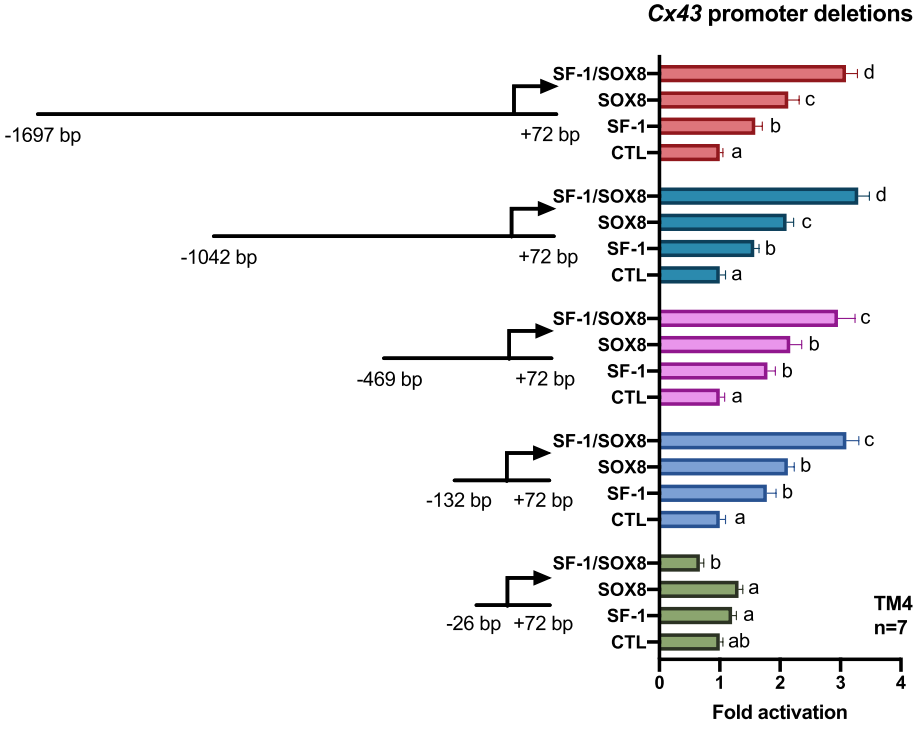

Fig. 2. Localization of the regulatory elements involved in the cooperation between SOX8 and SF-1 to activate the *Cx43* promoter. TM4 Sertoli cells were co-transfected with an empty expression vector (CTL) or expression vectors for SOX8 and/or SF-1 along with 5′ deletion constructs of the mouse *Cx43* promoter. The number of experiments, each performed in triplicate, is indicated. Results are shown as fold activation over control (CTL) (±SEM). Statistical analyses were performed using a two-way ANOVA followed by a Tukey multiple comparisons test (different letters: $P < 0.05$).

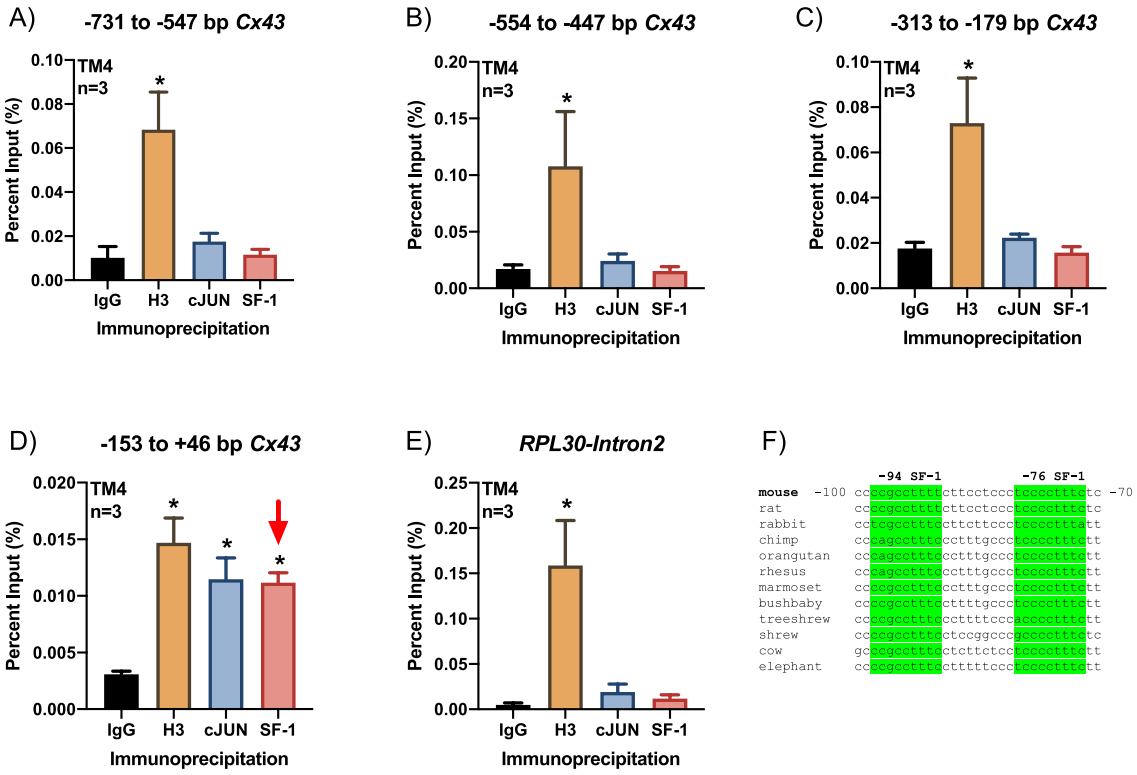

Fig. 3. Recruitment of SF-1 to the proximal region of the mouse *Cx43* promoter. Chromatin immunoprecipitation (ChIP) assays for SF-1 recruitment on the *Cx43* promoter were performed with TM4 Sertoli cells. Indicated *Cx43* promoter regions were amplified following ChIP assays. Recruitment using a normal rabbit IgG was used as a negative control. Histone H3 as well as cJUN were used as the positive controls for protein-DNA complexes and recruitment to the proximal region of the *Cx43* promoter, respectively. Amplification of a region of the second intron of *Rpl30* was used as a negative control for recruitment of SF-1 to DNA (E). Results are presented as percentages of input (±SEM). Statistical analyses were performed using an ANOVA, followed by a Bonferroni multiple comparisons' test. Statistically significant differences from control (IgG) are indicated by an asterisk (*$P < 0.05$). The proximal region of the *Cx43* promoter was investigated for SF-1 conserved regulatory elements using the CONTRAV3 platform [33] (F).

the *Cx43* promoter according to the CONTRAV3 platform [33] (Fig. 3F). As reported previously [22], the transcription factor cJUN was also recruited to the proximal region of the *Cx43* promoter according to the chromatin immunoprecipitation assays (Fig. 3D). Amplification of a region of the second intron of *Rpl30* served as a negative control for SF-1 and cJUN DNA recruitments (Fig. 3E).

3.4. Functional cooperation between SOX9 and Nur77 activates the mouse Cx43 promoter

To determine if other orphan nuclear receptors could cooperate with SOX transcription factors to activate the *Cx43* promoter in Sertoli cells, co-transfection assays were performed using the full-length *Cx43* promoter/luciferase reporter construct along with expression plasmids for Nur77, Nurr1, NOR1 and SOX8/9 (Fig. 4). Only the combination between Nur77 and SOX9 led to a cooperative activation of the *Cx43* promoter by 2.28 folds (Fig. 4D). Interestingly, over-expressions of Nur77 and SOX8 resulted in activation of the *Cx43* promoter by 1.94 folds in TM4 Sertoli cells (Fig. 4A). Using publicly available RNA-Seq data [30], we determined the expression profiles for *Sox* members, *Sf-1*, *Nur77* and *Cx43* according to different postnatal stages of mouse Sertoli cells (Fig. 4G). Although *Cx43* expression decreased from postnatal day 5 (P5) to postnatal day 10 (P10), its expression remained relatively constant from P10 to adulthood in Sertoli cells. Interestingly, the expressions of *Sf-1* and *Nur77* are correlated with *Cx43* according to post-natal development of Sertoli cells (Pearson $r = 0.8846$ and 0.9874, respectively). Although the expressions of *Sox8* and *Sox9* are poorly correlated with *Cx43*, these transcription factors may be functionally redundant to cooperate with *Sf-1* and *Nur77* to regulate *Cx43* expression.

To better define the promoter region involved in SOX9 and Nur77 dependent activation, 5′ deletions of the mouse *Cx43* promoter were evaluated for SOX9 and/or Nur77 responsiveness in TM4 Sertoli cells. As indicated in Fig. 5, a significant activation of the *Cx43* promoter was observed following co-transfection of SOX9 and Nur77 along with 5′ deletion constructs from −1697 to −132 bp. However, deletion from −132 to −26 bp resulted in a loss of *Cx43* promoter cooperative activation by SOX9 and Nur77, even though SOX9 dependent activation was maintained. These results suggest that DNA regulatory elements for the cooperation between SOX9 and Nur77 may be located between −132 and −26 bp of the *Cx43* promoter.

4. Discussion

Different types of connexins have been characterized in the somatic cells of the testis. In Sertoli cells, although the Cx43, Cx33, Cx32 and Cx26 proteins were detected [2,34], only the expression of Cx43 was found to be necessary for proper differentiation [35]. Sertoli cell-specific *Cx43* knockout mice are also known to have disrupted tight junctions in the blood-testis barrier [36–38]. In addition, the reduction or absence of *Cx43* expression from Sertoli cells leads to azoospermia and Sertoli cell-only syndrome [39], and is associated with an increased predisposition to testicular cancer among infertile men [40,41]. Therefore, a better understanding of the mechanisms of transcriptional regulation of *Cx43* in Sertoli cells could help to better define its involvement in certain pathophysiological conditions.

TM4 Sertoli cells retain the expression of genes important for the sex determination pathway, namely SOX9 [42], and also express SOX4. In

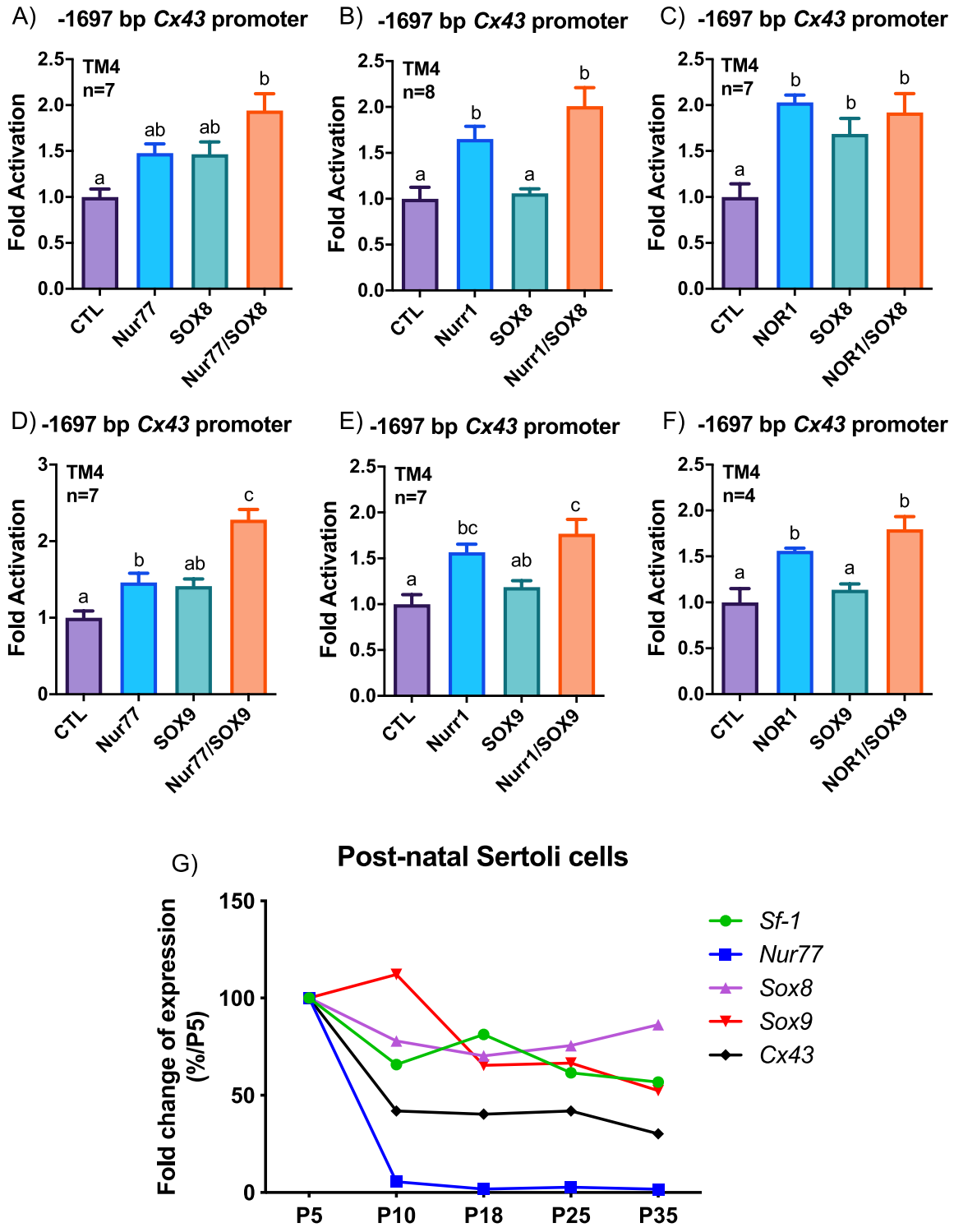

Fig. 4. Effects of the over-expressions of orphan nuclear receptors and SOX transcription factors on the full-length *Cx43* promoter luciferase reporter (−1697 bp to +72 bp) in TM4 Sertoli cells: Nur77/SOX8 (A), Nurr1/SOX8 (B), NOR1/SOX8 (C), Nur77/SOX9 (D) Nurr1/SOX9 (E) and NOR1/SOX9 (F). The control (CTL) corresponds to transfection of the full-length *Cx43* promoter reporter plasmid with an empty expression vector. Results are shown as fold activation over control (CTL) (±SEM). Statistical analyses were performed using a two-way ANOVA followed by a Tukey multiple comparisons test (different letters: $P < 0.05$). Following analysis of publicly available RNA-Seq datasets (NCBI SRA: PRJNA255976) [30], the expression profiles of *Sox* members, *Sf-1*, *Nur77* and *Cx43* are presented for 5 stages of mouse Sertoli cells postnatal development (G). Results are presented as percent fold change of P5 expression.

addition, we confirmed the expression of Cx43 in TM4 Sertoli cells [23], as demonstrated previously [2]. This cell line is therefore a suitable model for the characterization of the transcriptional regulation of *Cx43* in mouse Sertoli cells. As indicated in publicly available datasets, *Sox9* is highly expressed in post-natal Sertoli cells, while *Cx43* expression remains relatively constant from postnatal day 10 (P10) to adulthood. Interestingly, the expressions of *Sf-1* and *Nur77* are correlated with *Cx43* during post-natal development of Sertoli cells and may be interesting

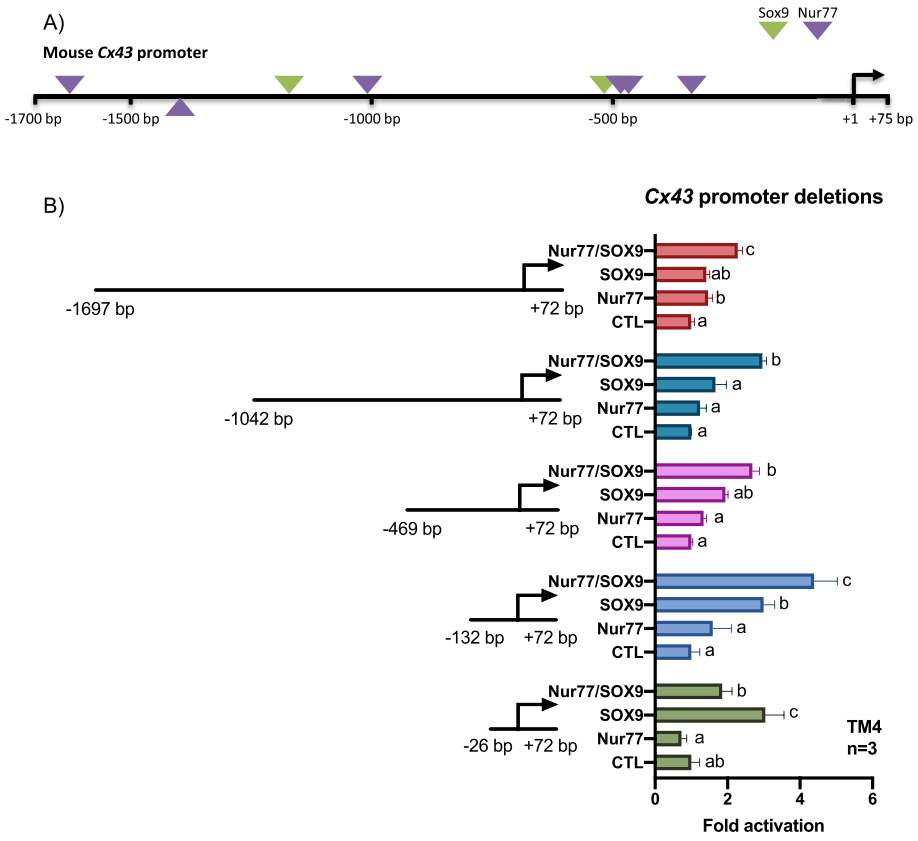

Fig. 5. Localization of the regulatory elements involved in the cooperation between SOX9 and Nur77 to activate the *Cx43* promoter. Regulatory elements for SOX9 and Nur77 within the *Cx43* promoter region have been identified using the JASPAR CORE 2018 database (A). TM4 Sertoli cells were co-transfected with an empty expression vector (CTL) or expression vectors for SOX9 and/or Nur77 along with 5′ deletion constructs of the mouse *Cx43* promoter (B). The number of experiments, each performed in triplicate, is indicated. Results are shown as fold activation over control (CTL) (±SEM). Statistical analyses were performed using a two-way ANOVA followed by a Tukey multiple comparisons test (different letters: $P < 0.05$).

partners for SOX dependant regulation of gene transcription. However, further investigation will be required to better define the impact of *Cx43* expression on cell-to-cell communication during prepubertal to adult testis development.

Although individual members of the SOX family have no effect when transfected alone, we have shown that the cooperation between SOX8 and SF-1 involves regulatory elements located within the −132 to −26 bp region of the *Cx43* promoter in TM4 Sertoli cells. This cooperation may rely on highly conserved SF-1 regulatory elements located at −94 and −76 bp in the proximal region of the mouse *Cx43* promoter. Interestingly, the proximal region of the *Cx43* promoter (up to −100 bp from its transcription start site) is highly conserved among mammals [43]. This region seems to be very important for the regulation of *Cx43* expression in Sertoli cells. Indeed, we have shown previously that SOX8 cooperates with cJUN to regulate the activity of the mouse *Cx43* promoter through the same region [22]. Cooperation between SOX8 and cJUN was expected due to their previously reported physical interaction [44]. Interestingly, only SOX8 and SOX9, but not SOX4, can physically interact with SF-1 [9,20], hence supporting the potential requirement of protein-protein interaction for *Cx43* promoter activation. Moreover, use of a dominant negative expression construct of SOX8, lacking the transactivation domain and interacting region for coactivators, resulted in loss of synergistic activation with SF-1 of the *Cx43* promoter. However, others have shown that the HMG box of SOX8 (amino acids 99–167) rather interacts with SF-1 to regulate the *Amh* promoter activity *in vitro* [9]. As an alternative, other transcription factors may serve as a bridge between SF-1 and SOX8. Indeed, others have shown that SF-1 physically interacts with cJUN [45,46] as well as SOX8 and SOX9 [9,20] and may bring these transcription factors together on the *Cx43* promoter. Therefore, regulation of *Cx43* promoter activity may not only require DNA binding but may also involve the recruitment of transcription factors by protein-protein interaction.

The chromatin immunoprecipitation assay (ChIP) was not performed for SOX8 due to a low amount of this protein in the Sertoli cells [22,47]. Although we could not confirm a direct binding of SOX8 to the proximal region of the *Cx43* promoter, its recruitment may rather involve an interaction with SF-1, as reported previously [9]. Thus, SF-1 and SOX8 may participate in the formation of a major complex within the −132 to −26 bp region of the mouse *Cx43* promoter to activate its expression within Sertoli cells. However, further investigation will be required to better define how these transcription factors participate in such a complex with AP-1 members to regulate *Cx43* expression.

In mouse 3T3-L1 cells, the *Cx43* promoter has been suggested to be directly regulated by the orphan nuclear receptor Nur77 during inhibition of adipocyte differentiation [48]. However, such Nur77 dependant regulation of *Cx43* promoter activity could not be observed in TM4 Sertoli cells and may involve other regulatory partners as we reported with SOX9. Furthermore, we confirmed that the proximal region of the *Cx43* promoter (between −132 and −26 bp) may be required for cooperative activation by SOX9 and Nur77. Since SOX9 dependant activation was maintained following deletion of the *Cx43* promoter to −26 bp, its regulatory element may be located downstream, whereas the Nur77 regulatory element may be located upstream, of −26 bp along the *Cx43* promoter. Interestingly, the expression of orphan nuclear receptors such as Nur77, Nurr1 and LRH-1 are highly induced by FSH in rat Sertoli cells [49]. In addition, our results suggest that SF-1 and LRH-1 may be functionally redundant to cooperate with SOX8 for upregulation of *Cx43* expression in Sertoli cells. However, SF-1, but not LRH-1, is being expressed in primary and TM4 Sertoli cells [50].

In conclusion, we have shown that a functional cooperation between the transcription factors SF-1 and SOX8, as well as SOX9 and Nur77, activates *Cx43* expression within TM4 Sertoli cells. In addition, such synergy involving SF-1 depends on its recruitment to the proximal region of the mouse *Cx43* promoter. DNA regulatory elements for SF-1 and SOX8 recruitment have never been identified before and may also involve an indirect binding to the mouse *Cx43* promoter. Hence, our

data suggest that the cooperation between SOX8 and SF-1 may be important for optimal expression of *Cx43* within Sertoli cells of the mouse testis.

Disclosure

The authors declare that there is no conflict of interest that would prejudice their impartiality.

Funding

This work was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada (grant number: 386557 to L.J.M.) and the New Brunswick Innovation Foundation (NBIF) (grant number: RIF_2019_014 to L.J.M.).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Roxanne Couture: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, preparation. **Luc J. Martin:** Conceptualization, Supervision, Validation, Formal analysis, Visualization, Funding acquisition, Writing - review & editing.

Acknowledgements

We would like to thank Drs. Jacques Drouin, Luc Bélanger, Michael Wegner and James Wells for generously providing expression plasmids used in this study.

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