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Review

Regulation of connexin expression by transcription factors and epigenetic mechanisms ☆

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ABSTRACT

Gap junctions are specialized cell–cell junctions that directly link the cytoplasm of neighboring cells. They mediate the direct transfer of metabolites and ions from one cell to another. Discoveries of human genetic disorders due to mutations in gap junction protein (connexin [Cx]) genes and experimental data on connexin knockout mice provide direct evidence that gap junctional intercellular communication is essential for tissue functions and organ development, and that its dysfunction causes diseases. Connexin-related signaling also involves extracellular signaling (hemichannels) and non-channel intracellular signaling. Thus far, 21 human genes and 20 mouse genes for connexins have been identified. Each connexin shows tissue- or cell-type-specific expression, and most organs and many cell types express more than one connexin. Connexin expression can be regulated at many of the steps in the pathway from DNA to RNA to protein. In recent years, it has become clear that epigenetic processes are also essentially involved in connexin gene expression. In this review, we summarize recent knowledge on regulation of connexin expression by transcription factors and epigenetic mechanisms including histone modifications, DNA methylation, and microRNA. This article is part of a Special Issue entitled: The communicating junctions, roles and dysfunctions.

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1. Introduction

Gap junctions are specialized cell–cell junctions that directly link the cytoplasm of neighboring cells. They mediate the direct transfer of metabolites and ions from one cell to another. Therefore, it has long been hypothesized that gap junctional intercellular communication plays a crucial role in the maintenance of homeostasis, morphogenesis, cell differentiation, and growth control in multicellular organisms. Discoveries of human genetic disorders due to mutations in gap junction protein (connexin [Cx]) genes and experimental data on connexin knockout mice provide direct evidence that gap junctional intercellular communication is essential for tissue functions and organ development, and that its dysfunction causes diseases. Connexin-related signaling also involves extracellular signaling (hemichannels) and non-channel intracellular signaling.

Connexin proteins are named after their specific molecular weight in kDa (for instance, Cx43 has a mobility of 43 kDa). Twenty-one human genes and 20 mouse genes for connexins have been identified [1]. Their genes have been classified into 5 groups (alpha, beta, gamma, delta, and epsilon) based on sequence homology and thus the genes are named accordingly (for instance Cx43, which is the first connexin of the alpha-group, is coded by GJA1) (http://www.genenames.org/genefamilies/GJ). Each connexin shows tissue- or cell-type-specific expression, and most organs and many cell types express more than one connexin (Table 1). Some connexins, such as Cx32 and Cx43, are expressed in cells of many types, but others are expressed in very limited organs and cells. Even in the same tissue, the expression pattern of each connexin shows cell-type specificity and developmental changes, suggesting the presence of distinct but tight control mechanisms for regulation of connexin gene expression. For example, in the adult mouse heart tissue [2,3], Cx43, encoded by the *Gja1* gene, is expressed in all the cardiac components excluding the sinoatrial node (SAN) and atrioventricular node (AVN), the His bundle, and the proximal parts of the bundle branches (BBs). On the other hand, Cx40, encoded by *Gja5*, expression is restricted to the atrial myocytes, the AV node, and the His–Purkinje system. Similarly, Cx45, encoded by *Gjc1*, is restricted to the SAN and AVN, around the His bundle, and the most peripheral regions of the interventricular septum. Cx30.2, encoded by *Gjd3*, is expressed in the SAN and AVN, and to a lesser extent in the His bundle and its branches. Cx30.2 contributes to slow down impulse propagation in the AVN, and to limit the number of beats conducted from atria to ventricles. Cx30, encoded by *Gjb6*, is functionally expressed, in low abundance, in the SAN.

Cell coupling via gap junctions is dependent on the specific pattern of connexin gene expression [4]. This pattern of gene expression is altered during development and in several pathological conditions resulting in changes of cell coupling and probably connexin hemichannel function [5]. Like other genes, connexin expression can be regulated at many of the steps in the pathway from DNA to RNA to protein, i.e., transcriptional control, RNA processing control, RNA transport and localization control, translational control, mRNA degradation control, and protein activity control [6,7]. More recently the contributions of epigenetic and post-transcriptional mechanisms

Table 1
Human gap junction protein (connexin) genes.

Approved symbol	Approved name	Synonyms	Chromosome	Major expressed organ or cell types
GJA1	Gap junction protein, alpha 1, 43 kDa	Cx43	6q22-q23	Many cell types
GJA3	Gap junction protein, alpha 3, 46 kDa	Cx46	13q12.11	Lens
GJA4	gap junction protein, alpha 4, 37 kDa	Cx37	1p35.1	Endothelium, granulosa cells, lung, skin
GJA5	Gap junction protein, alpha 5, 40 kDa	Cx40	1q21.1	Cardiac atrium and conduction system, endothelium
GJA8	Gap junction protein, alpha 8, 50 kDa	Cx50	1q21.1	Lens
GJA9	Gap junction protein, alpha 9, 59 kDa	Cx59, Cx58	1p34	–
GJA10	Gap junction protein, alpha 10, 62 kDa	Cx62, mouse Cx57	6q15-q16	Retinal horizontal cells
GJB1	Gap junction protein, beta 1, 32 kDa	Cx32	Xq13.1	Hepatocytes, secretory acinar cells, Schwann cells
GJB2	Gap junction protein, beta 2, 26 kDa	Cx26	13q11-q12	Cochlea, placenta, hepatocytes, skin, pancreas, kidney, intestine
GJB3	Gap junction protein, beta 3, 31 kDa	Cx31	1p34	Cochlea, placenta, skin
GJB4	Gap junction protein, beta 4, 30.3 kDa	Cx30.3	1p35-p34	Skin, kidney
GJB5	Gap junction protein, beta 5, 31.1 kDa	Cx31.1	1p34.3	Skin
GJB6	Gap junction protein, beta 6, 30 kDa	Cx30	13q12	Astrocytes, cochlea
GJB7	Gap junction protein, beta 7, 25 kDa	Cx25	6q15	–
GJC1	Gap junction protein, gamma 1, 45 kDa	Cx45	17q21.31	SAN, AVN, smooth muscle cells, neurons
GJC2	Gap junction protein, gamma 2, 47 kDa	Cx47, Cx46.6,	1q41-q42	Oligodendrocytes, spinal cord, lymphatics
GJC3	Gap junction protein, gamma 3, 30.2 kDa	Cx30.2	7q22.1	Brain, spinal cord, Schwann cells
GJD2	Gap junction protein, delta 2, 36 kDa	Cx36	15q13.1	Neurons, pancreatic β-cells
GJD3	Gap junction protein, delta 3, 31.9 kDa	Cx31.9, Cx30.2	17q21.1	SAN, AVN
GJD4	Gap junction protein, delta 4, 40.1 kDa	Cx40.1	10p11.22	–
GJE1	Gap junction protein, epsilon 1, 23 kDa	Cx23	6q24.1	–

like DNA methylation, histone modifications, and microRNAs have been identified in regulation of connexin expression [8]. In the previous review [9], we focused on the structure of connexin genes, and transcriptional factors and biological substances that regulate connexin expression. In the current review, we will add new information on regulation of connexin expression by transcription factors and epigenetic mechanisms.

2. Gene structure of gap junction proteins (connexins)

Initial studies seemed to show that the general genomic structure of connexin genes is rather simple, i.e., a 5′-untranslated region (5′-UTR) on exon 1 is separated from the complete connexin coding region and the subsequent 3′-untranslated region (3′-UTR), both located on exon 2. However, current data on genomic organization of various connexin genes refutes this simplicity. First, many different splice isoforms of several connexin genes have thus far been identified, indicating that different 5′-UTRs can be spliced in a consecutive and/or alternate manner possibly due to alternate promoter usage. However, it should be emphasized that these transcript isoforms vary only in their untranslated, mostly 5′-UTRs that leave the coding region unaltered. Second, the coding region can be interrupted by introns, e.g., mouse Cx36 (*Gjd2*) [10], Cx39 (*Gjd4*) [11], Cx57 (*Gja10*) genes [12], and human Cx59 (*GJA9*) [13]. We will discuss the gene structure of several connexin genes in some detail in the following sections.

2.1. Cx43 (GJA1)

Cx43 gene was originally described as consisting of two exons, one containing most of the 5′-UTR and the other containing the protein sequence and 3′-UTR [14–17]. However, Pfeifer et al. [18] reported that in mice there are four additional exons, all of which code for novel 5′-UTRs. They showed that altogether six exons, five of which code for 5′-UTRs (exon 1A~1E), one for the coding region (exon 2), and three alternate promoter regions (P1-P3) are present in the mouse Cx43 gene, and that four of the six exons (exon 1A–1C, exon2) found in the mouse are also present in the rat. No equivalents of mouse exons 1D or 1E were found in the rat. In the human, however, no alternate 5′-UTR has been found so far.

Pfeifer et al. [18] also showed that in the mouse, nine different Cx43 mRNAs (transcripts E1As/E2, E1A/E2, $E1A_L/E2$, E1A/E1E/E2, E1Bs/E2, E1Bs/E1D/E2, $E1B_L/E2$, E1C/E2, E1C/E1D/E2) are generated by differential promoter usages and alternative splicing mechanisms. In rats, six different Cx43 mRNA species (transcripts E1As/E2, E1A/E2, $E1A_L/E2$, E1B/E2, E1Cs/E2, $E1C_L/E2$) were similarly found. In the mouse Cx43 gene, in addition to the promoter P1, which was previously known to be a Cx43 promoter, two additional promoters downstream of promoter P1 were found; promoter P2 is located within exon 1A, and promoter P3 is located in the intron just upstream of exon 1C. In the heart, promoter P1 is active throughout the organ. Promoter P2 is active in the atrium and septum but not in the ventricle. Promoter P3 is functional only in ventricles and not in atria and septum. Alternative splicing and exon choice seem to be cell-type specific, because transcripts containing exon 1B preferentially skip exon 1D in the septum, whereas in the atrium exon 1D is included in about half of the transcripts.

Bierhuizen et al. [19] analyzed the expression of Cx43 RNA isoforms in mouse calcineurin-induced cardiac hypertrophy. The expression profiles for the different RNA Cx43 isoforms are very similar between wild-type and calcineurin-induced hearts not only in the left ventricle but also in the other parts of the heart.

Carystinos et al. [20] have proposed that the P2 promoter mediates upregulation of Cx43 expression through the Ras signaling pathway and the putative consensus sequence, AGTTC(A/C)A(T/C)CA, was found in the human, mouse and rat Cx43 gene. They showed that this element is recognized by a protein complex that includes c-Myc and HSP90.

2.2. Cx40 (GJA5)

Concerning Cx40 mRNA, two different transcripts are found in the human [21], three different transcripts in the mouse [22], and one transcript in the rat have been found. More importantly, these different transcripts are transcribed in a tissue-specific manner in the human and mouse.

Dupays et al. [21] reported that human Cx40 gene contains at least three exons, 1A, 1B and 2, which are present in two lineage-specific variants: transcript 1A and transcript 1B are derived from alternative 5′ non-coding exons (exon 1A and 1B). Each of two exons is transcribed independently [transcript 1A (E1A/E2) and transcript 1B (E1B/E2)]. These transcripts are transcribed in a cell-type-specific manner; e.g., human umbilical cord vein endothelial cells (HUVEC) express only transcript 1A, while the human choriocarcinoma cell lines BeWo, JAR, and JEG-3 and purified cytotrophoblasts from first-trimester human placenta express only transcript 1B. In the human heart, however, both transcripts were found to be expressed in various regions, i.e., left atrium, right atrium, left ventricle, and right ventricle. Quantitative real-time RT-PCR analysis showed that transcript 1B is always less abundant than transcript A in these different regions in the heart: for example, transcript 1B was 3- and 28-fold less abundant than transcript 1A in the left atrium and left ventricle.

In the mouse Cx40 gene, Anderson et al. [22] indentified three 5′-UTR exons, exon 1A, 1B, and AS, and three transcripts (E1A/E2, E1B/E2, E1B/AS/E2). A transcript containing exon AS is nearly absent in mouse embryo and heart, but it is especially abundant in the esophagus. On the other hand, transcripts that include exon 1a were reported to be ubiquitous in all the tissues tested. In addition, Bierhuizen et al. [19] analyzed the expression of Cx40 RNA isoforms in mouse calcineurin-induced cardiac hypertrophy. RNA consisting of E1A/E2 sequences could not be detected at all in wild type nor calcineurin-induced atrial samples. The RNA isoforms consisting of E1B/E2 or E1B/AS/E2 sequences are detectable in control RNA, but are largely reduced in calcineurin-induced atrial RNA.

In the rat, no alternative Cx40 transcripts have so far been reported [23]. Human Cx40 exon 1A is orthologous to the mouse Cx40 exon 1B and rat Cx40 exon 1.

Tao and Valdimarsson [24] recently reported that zebrafish Cx45.6 (the ortholog of mammalian Cx40) shows six isoforms of alternative 5′UTRs which are generated from multiple promoter usage and alternative pre-mRNA splicing. Cx45.6 undergoes tandem alternative splicing, which produces transcripts only differing by 3 nucleotides. Their work is the first study that has demonstrated tandem alternative pre-mRNA splicing in the connexin gene family.

2.3. Cx32 (GJB1)

Miller et al. [25] first reported the structure of the rat Cx32 gene and showed that the 5′-UTR of the transcript in the liver contains a 6.1-kb intron and that transcription starts at non-coding exon (in this review, the nomenclature used for an exon localized at most 5′ regions is exon 1) upstream of the intron, while the complete coding sequence is not interrupted by an intron but contained within one exon (in this review, the nomenclature used for this exon is exon 2). It is now known that multiple alternatively spliced transcripts of Cx32 gene exist in mammalian species, i.e., two different transcripts in the rat [26,27] and human [28], and three different transcripts in the cow [29] and mouse [30]. It can be generalized that in the hepatocyte and secretory acinar cell, Cx32 mRNA is made from the promoter 1 (P1) upstream of exon 1 and in Schwann cells from an alternative promoter 2 (P2) upstream of exon 1B that is located upstream of the coding exon (exon 2). Therefore, promoter P2 is called nerve-

specific promoter (in this review, the nomenclature used for an exon downstream of the nerve-specific promoter of Cx32 gene is exon 1B). In cattle and mice, exon 1A is localized between exon 1 and exon 1B.

The promoter P1 contains putative binding sites not only for cell type-independent (ubiquitous) transcription factors, such as binding sites for Sp1/Sp3 [31–33], nuclear factor 1 (NF-1) [34], and Yin Yang 1 (YY1) [33], but also for cell-type-dependent transcription factors including hepatocyte nuclear factor-1 (HNF-1) [31,34,35]. The nerve-specific promoter P2 also includes binding sites for cell-type-specific transcription factors such as Sox10 and early growth response gene-2 (Egr2/Knox20) [36,37]. Each transcription factor will be described below.

2.4. Cx26 (GJB2)

In contrast to other connexin genes described above, thus far, only two exons, i.e., non-coding exon 1 and exon 2 containing complete connexin coding region and the subsequent 3′-UTR, are known to be present in the mammalian Cx26 gene. In the mouse Cx26 gene, the length is 234 bp for exon 1 and 3.8 kb for intron 1 [34]. Similarly, in the human Cx26 gene, the length is 160 bp for exon 1 and 3148 bp for intron 1 [38]. In the promoter P1 upstream of exon 1, several GC boxes for Sp1/Sp3 binding sites and a TTAAAA box are identified in the mouse [34] and human [38,39] Cx26 genes.

2.5. Cx31 (GJB3)

Three exons (exons 1A, 1B, and 2) have so far been identified in the mouse Cx31 gene. The two exons that comprise the 5′-UTR, exons 1A and 1B, lie ~3.7 kb and 2.3 kb upstream of exon 2 (coding exon), respectively [22,40]. Anderson et al. [22] described exon 1A, a novel exon, which appears to be transcribed from previously unknown promoters. They showed that transcripts that include exon 1A are widely distributed in adult tissues, including the skin, but are absent from the brain, and that multiple transcription start sites are present in exon 1A.

Mouse exon 1B was cloned and described by Henneman et al. [41]. A proximal promoter region extending to 561 bp upstream of mouse exon 1B serves as a basal promoter in both mouse embryonic stem (ES) cells and a mouse keratinocyte-derived cell line. The rat exon 1 shows high homology (93%) with mouse exon 1B [40]. Two transcription start sites are identified in exon 1 in the rat. However, in the human Cx31 gene, only the second exon, which contains complete coding region and a part of intron, has been characterized [42].

Both mouse Cx31 exon 1B and rat Cx31 exon 1 are preceded by a TATA-less promoter region. No GC boxes (which may control transcriptional initiation in TATA-less promoters) via binding to the ubiquitous transcription factor Sp1 were found in the (basal) promoter region [40,43]. In rat Cx31 promoter region (935 bp upstream of the 5′ flanking region of exon 1), five putative GATA-2/GATA-3 binding sites, and putative binding sites for NFκB, CCAAT-box, cEBPα/CEBβ, c-AMP responsive element, and multiple E-box/E-box are found. Despite the presence of multiple binding sites for GATA factors in the putative promoter region of rat Cx31 gene, cotransfection experiments with GATA-3 expression had no influence on the promoter activity of Cx31. At further upstream of basal promoter region (between −3000 and −2200 bp upstream), positive cell-specific regulatory element is found in the rat Cx31 gene [40].

In the mouse, expression of Cx31 in keratinocytes and ES cells is regulated by different cis-regulatory elements and differs in its requirements for the intron in situ [43]. A region between 561 and 841 bp upstream of mouse Cx31 exon 1B is essential and sufficient for substantial transcription of Cx31 in mouse keratinocyte-derived Hel37 cells, whereas an intron between exon 1B and exon 2 enhances expression of Cx31 in ES cells but not in keratinocyte-derived cells. Splicing is shown to be required for intron-dependent expression enhancement of Cx31 in ES cells because mutations in the splice donor site that prevent transcript processing cause decreased expression of Cx31. The enhancing effect of the intron of the rat Cx31 gene was also found in the mouse keratinocyte line Hel37 as well as in the choriocarcinoma cell line Rcho-1 [40].

2.6. Cx30 (GJB6)

Essenfelder et al. [44] reported that six different exons are present in the human Cx30 gene. They showed that exon 1 to exon 5 are non-coding exons while exon 6 is a coding exon, and that some of the Cx30 exons can be alternatively spliced, so that the 5′ non-coding region of Cx30 transcripts is highly variable. They found that in hair follicle keratinocytes, at least four different splicing variants are present and only exon 5 is present in all transcripts, whereas exon 3, which has been described in human brain cDNA, is absent from Cx30 transcripts from epidermis. Their data suggest that in epidermis, Cx30 transcription starts at exon 1, whereas in cells from the nervous system transcription would start from exon 3.

In the promoter region of the human Cx30 gene, upstream of exon 1, a TATA motif (TTAAAA), several potential binding sites for Sp1, and consensus sequence (CGCCCCCGC) for the early growth response gene product (Egr)-binding are present [44].

2.7. Cx45 (GJC1)

The mouse Cx45 gene is composed of five exons (exons1A, 1B, 1C, 2, and 3), two of which (exons 1A and 1B) were only very recently reported [22]. Exons 1A, 1B, 1C, and 2 contain only 5′ untranslated sequences, and exon 3 contains the remaining 5′-UTR, entire coding sequence and 3′-UTR. Each of exon 1A, 1B, and 1C is spliced to exon 2 and exon 3. A transcript that directly starts at exon 2 is also present [22,45]. Thus, various transcripts (E1A/E2/E3, E1B/E2/E3, E1C/E2/E3, E2/E3) are generated. Transcription of Cx45 from each alternative promoter is tissue-specific, i.e., although transcripts containing exon 1A is nearly ubiquitous, exon 1B was found in trace amounts in colon RNA and Exon 1C was found in colon, bladder, lung, skeletal muscle, ovary, heart, and E14.5 embryo total RNA. Multiple upstream ORF are found in exon 1A and exon 1C [22,45].

Teunissen et al. [46] performed comparative analysis of the Cx45 gene between human and mice, and revealed conservation of E2 and E3 sequence between the two species.

Baldridge et al. [47] found a potential TATA box and two putative AP-1 binding sites in the 5′ region of the mouse Cx45 gene, but no functional analysis of the regulatory region of Cx45 has yet been performed.

The usage of alternative promoters to produce different 5′-UTR of connexin mRNAs has also been reported on mouse Cx46 (*Gja3*) and Cx47 (*Gjc2*) [22].

2.8. Cx36 (Gjd2), Cx39 (Gjd4), Cx57 (Gja10), and Cx59 (GJA9) genes, whose coding regions are interrupted by introns

The coding regions of *Cx36*, *Cx39*, *C*x57, and Cx59 genes have been shown to be located on two (or more) different exons. The coding region is interrupted by a 1.14-kb intron, which separates the first 71 bp, starting with ATG, from the rest of the coding region in the rat [48,49] and mouse [49] Cx36 genes. The coding region of the mouse Cx39 is also interrupted by a 1.5-kb intron, which separates exon 1, coding 21 amino acids, and exon 2, coding 343 amino acids [50]. Concerning mouse Cx57 gene, Hombach et al. [12] found that, at least in the retina, these most C-terminal amino acid residues were replaced after splicing with 12 different amino acid residues coded further downstream. As a result, 97.6% (480 amino acids) of the coding region of mouse Cx57 gene is located on exon 2, whereas the residual 2.4% (12 amino acids) is encoded on a third exon, which

is separated by an intron of about 4 kb. When connexin genes contain coding regions that are interrupted by introns, these coding regions have to be spliced properly in order to become translated. Otherwise alternative splicing (which has not, however, been demonstrated yet) would lead to a dramatic modification of the connexin coding region.

3. Transcriptional factors, biological substances, and signal transduction pathways that regulate expression of connexin genes

As described above, in most connexin genes, the basal (canonical, proximal) promoter P1 is located within 300 bp upstream of the transcription initiation site in exon 1. Within the region, binding sites for cell type-independent (ubiquitous) and -dependent transcription factors have been identified in several connexin genes. For example, the former include binding sites for TATA box-binding protein, Sp1/Sp3, and AP-1. The latter include binding sites for cardiac-specific transcription factors (Nkx2.5, Tbx5, Tbx2, Tbx3, GATA4 etc.) and HNF-1. A sharp distinction between cell type-independent (ubiquitous) and -dependent transcription factors is sometimes difficult to make. However, we adopt this classification for the convenience of description.

3.1. Cell type-independent (ubiquitous) transcription factors, biological substances, and signal transduction pathways that regulate connexin expression

3.1.1. Sp1

Sp1 is a ubiquitous transcription factor that has a DNA-binding domain that consists of three zinc fingers and recognizes the GC box. Sp-1 binding sites have been identified in connexin genes, and they appear to be a common important element in the basal transcriptional activity of several connexin genes, such as Cx32 [31–33], Cx40 [21,51–54], Cx43 [55–61], and Cx26 [39,62]. For example, Teunissen et al. [53,56] characterized the proximal promoters P1 of rat Cx40 (−175, +85) and Cx43 (−148, +281) genes, in which respectively five and four potential binding sites for Sp-family transcription factors were found. They showed that each of these sites contributes to promoter activities and binds both the transcription factors Sp1 and Sp3. They also demonstrated that both Sp1 and Sp3 activated the rat Cx40 and Cx43 promoters P1, and that random disruption of two of the Sp1/Sp3 binding sites almost completely abolished promoter activity of Cx40 genes.

Hernandez et al. [60] found that the Trichostatin A (TSA), an inhibitor of a histone deacetylase, induces Cx43 in human prostate cancer cells. They provided evidence that the integrity of both Sp1 and a run of AP-1 in a regulatory region between −234 and −287 from the transcription start site of the basal Cx43 promoter is essential for TSA-induced Cx43 promoter activity, showing that the Sp1/AP-1 responsive elements act in a synergy with the coactivator p300/CREB-binding protein (CBP) in TSA-mediated Cx43 transcription. This is accompanied by hyperacetylation of histones H4 surrounding the AP-1- and Sp1-responsive gene elements.

Villares et al. [61] reported that up-regulation of protease-activated receptor-1 (PAR-1) expression, seen in melanoma progression, mediates high levels of Cx43 expression and that Cx43 promoter activity is significantly inhibited in PAR-1-silenced cells. Chromatin immunoprecipitation studies showed a reduction in the binding of SP-1 and AP-1 transcription factors to the promoter of Cx43 after PAR-1 silencing.

Makino et al. [63] showed that high-glucose treatment causes a decrease in Cx40 protein expression in vascular endothelial cells and impairs endothelial capillary network formation, and that the hyperglycemia-induced decrease in Cx40 is associated with inhibited protein expression of Sp1.

Concerning Cx32, Bai et al. [32] identified two Sp1 binding sites in a basal promoter P1 localized between −179 bp and −134 bp in the rat Cx32 gene. Piechocki et al. [31] also showed that specific nuclear protein-DNA complexes that bound to Sp1 consensus sites within the rat Cx32 basal promoter element (nt −134 to −33) are formed using nuclear extracts from both types of cells. The basal promoter element of rat Cx32 gene (nt −134 to −33) is 1.4-fold more active in MH1C1 cells than in WB-F344 cells, whereas the entire promoter fragment (nt −754 to −33) is four-fold more active in MH1C1 cells. Specific nuclear protein-DNA complexes that bound to Sp1 consensus sites within the basal promoter P1 are formed using nuclear extracts from both MH1C1 rat hepatoma cells that express endogenous Cx32 and WB-F344 rat liver epithelial cells that do not. These data indicate that Sp1 is necessary for Cx32 promoter P1 basal activity, but that some other transcription factor(s) determine the cell-specific expression of Cx32.

3.1.2. Activator protein 1 (AP-1)

Activator protein 1 (AP-1) is composed of homo- or heterodimers of the Jun, Fos, activating transcription factor (ATF), and musculoaponeurotic fibrosarcoma (MAF) proteins via leucine zipper structure and regulates various responses of cells to stimuli. AP-1 binds to a consensus sequence [TGAC (T/G) TCA] in the promoter and induces transcription of the gene. One or more AP-1-binding sites have been identified in the mouse, rat, and human Cx43 proximal promoters P1 (approximately 150 nucleotides up- and downstream of the transcription initiation site) (the rat Cx43 promoter contains two AP-1-binding sites [56], whereas the mouse and human promoters each have one AP-1-binding site). The importance of AP-1 transcription factors in regulation of Cx43 expression in the onset of labor by allowing for an increase in myometrial muscle cell coupling has been demonstrated [17,58,64].

Mitchell and Lye [65] reported that dimers comprising Fos/Jun proteins conferred greater transcriptional activity than Jun dimers, with the Fra-2/JunB combination conferring the greatest activity, and that expression of Fra-2 increases earlier than other Fos family members and confers the highest transcriptional drive to the Cx43 promoter, suggesting that Fra-2 is a central component in the regulation of Cx43 expression during labor.

Tacheau et al. [66] found that TGF-beta1 induces Cx43 gene expression in normal murine mammary gland epithelial cells and that c-Jun/AP-1 pathway together with p38 and PI3K/AKT pathways is involved in mediating TGF-beta1-induced Cx43 gene expression.

Zi et al. [67] very recently reported that mitogen-activated protein kinase kinase 4 (MKK4) deficiency in cardiomyocytes causes Cx43 reduction and couples hypertrophic signals to ventricular arrhythmogenesis. They found that two AP-1 binding sites, (−47, -39 from the transcription start site) and (−122, -112 from the transcription start site), in the Cx43 promoter region are responsible for the MKK4-regulated Cx43 expression.

Negoro et al. [68] found that basic fibroblast growth factor (bFGF) up-regulates Cx43 transcription in urinary bladder smooth muscle cells via the extracellular signal regulated kinase (ERK) 1/2-AP-1 pathway. They showed that Cx43 promoter activity is significantly up-regulated upon stimulation of bFGF and that the effect of bFGF on Cx43 promoter is abolished by mutation or deletion of an AP-1 binding site (−49, −43) of the Cx43 promoter.

3.1.3. Cyclic AMP

It has long been known that cyclic AMP (cAMP) enhances gap junction formation and gap-junctional intercellular communication in many cell types. Concerning gap junctions made by Cx43, it is now understood that such enhancement by cAMP is achieved via at least two different mechanisms: initial rapid redistribution of Cx43 to the cell membrane, and later stimulation of Cx43 gene expression. Putative camp (C/EBP)-responsive element had been identified in the rat Cx43 proximal promoter over a decade ago [16]. Civitelli et al. [69] demonstrated that when rat Cx43 promoter P1 [including 1,339 bases 5′ from the transcriptional start site (+1) and 222 bases 3′]-luciferase transfected rat osteogenic sarcoma cells (UMR 106–01 cells) were

treated with 8Br-cAMP for 6 h, the amount of luciferase activity increased to 2 times the basal activity. van der Heyden et al. [70] also showed that dibutyryl-cAMP treatment enhances the activity of rat Cx43 promoter P1 (containing exon I and 1250 bp of upstream sequence)-luciferase, and that enhancement of Cx43 promoter activity by cAMP is an additional to that by the Wnt pathway, which will be discussed below, indicating that cAMP and Wnt signaling pathways act in parallel on the Cx43 promoter P1. However, to our knowledge, there are no reports showing that the putative cAMP-responsive element in the proximal Cx43 promoter is really needed for enhancement of Cx43 expression by cAMP or that protein complexes bind to the element. For example, de Montgolfier et al. [71] very recently reported that in the brook trout testis, while cAMP can stimulate the transactivation of the Cx43 promoter, it does not do this via the C/EBP binding domain since a mutation in this sequence fails to alter the transactivation of the Cx43 promoter.

3.1.4. Wnt pathway

Wnt genes encode a large family of secreted glycoproteins which play important roles in directing cell fate and cell behavior, not only during embryonic development and in adult life, but also in tumorigenesis [72]. Wnt proteins are thought to act via the Frizzled class of cell-surface proteins. Receptor activation leads to inhibition of glycogen synthase kinase 3β (GSK3β), resulting in stabilization and accumulation of nonphosphorylated β-catenin within the cytosolic compartment. Increased abundance of this pool is associated with entry of β-catenin into the nucleus, where members of the T cell factor/lymphocyte enhancer binding factor (TCF/LEF) family of transcription factors physically interact with β-catenin. In the nucleus, before Wnt signaling, TCF/LEF members bind to DNA with sequence specificity in promoter and enhancer regions of target genes, and along with Groucho and C-terminal binding protein (CtBP), often repress gene expression. Nuclearly localized β-catenin/TCF complexes are supposed to activate transcription of target genes by binding to the specific consensus sequence A/TA/TCAAAG, known as the TCF/LEF binding site.

van der Heyden et al. [70] performed a computer search for TCF/LEF regulatory elements in the rat Cx43 promoter P1 and found that this promoter contains two TCF/LEF binding consensus sequences in opposite orientations, i.e., TCF/LEF site 1 is located at −1,394 bp while site 2 is located at −714 bp with respect to the transcription start site, and a third consensus sequence is found in the first intron following a non-translated short exon. Two similar TCF/LEF motifs are also found in the human and mouse Cx43 promoters partly at similar positions, and a third one is located in the first intron [17,73].

In fact, several studies have suggested that Cx43 acts as a functional target of Wnt1 signaling. van der Heyden et al. [70] investigated the effects of Wnt1 overexpression on gap-junctional communication in the rat neural-crest-derived cell line PC12. They found that Wnt1-expressing clones display an increased electrical and chemical coupling, which coincides with an increased expression of Cx43 mRNA and protein, whereas other connexins, Cx26, Cx32, Cx37, Cx40, and Cx45, are not upregulated. They also showed in transient transactivation assays in P19 EC cells that Wnt1 and Li^+, an ion that mimics Wnt signaling, increase transcription from the rat Cx43 promoter containing exon I and 1250 bp of upstream sequence, potentially via TCF/LEF binding elements. Ai et al. [74] also reported that neonatal rat cardiomyocytes respond to Li^+ by accumulating the effector protein β-catenin and by inducing Cx43 mRNA and protein markedly, and that by transfecting a Cx43 promoter P1-reporter gene construct into cardiomyocytes, the inductive effect of Wnt signaling is transcriptionally mediated.

Xia et al. [75] recently demonstrated that β-catenin directly binds to the Cx43 promoter, stimulating Cx43 expression and functional gap junctions between osteocytes. They reported three findings: (i) fluid flow shear stress and prostaglandin E_2 (PGE_2) activate phosphoinositide-3 kinase (PI3K)/Akt signaling, which increases the levels of Cx43 expression and gap junction activity; (ii) the independent activation of PI3K/Akt and cAMP protein kinase A signaling by PGE_2 converges through the downstream inactivation of GSK-3β and subsequent activation of β-catenin pathway; (iii) PGE2-induced PI3K/Akt signaling leads to inactivation of GSK-3β followed by nuclear translocation and accumulation of β-catenin, which associates with the Cx43 promoter to transcriptionally regulate Cx43 expression.

3.2. Cell type-dependent transcription factors and biological substances that regulate connexin expression

In addition to the maintenance of basal level of connexin transcription by the cell type-independent (ubiquitous) transcription factors such as Sp1 and AP-1, the tissue-specific expression of different connexin genes depends on additional cell type-specific activators or repressors. Although the molecular mechanisms that regulate cell type-specific transcription of each connexin gene are still not well understood, recent studies have led to a better understanding of the roles of some cell-type-dependent transcription factors in regulation of connexin expression. For example, a great deal of knowledge has been accumulated on the crucial roles of transcription factors in cardiac development including development of the cardiac conduction system and cardiac connexin (Cx30.2, Cx40, Cx43, Cx45) expression in the heart [76–79].

3.2.1. Homeobox proteins

A homeobox is about 180 base pairs long and encodes a 60-aa domain (the homeodomain) which when expressed (i.e. as protein) can bind DNA. Homeobox-containing genes encode transcription factors (homeobox proteins) that typically switch on/off cascades of other genes. The homeodomain binds DNA in a sequence-specific manner. However, the specificity of a single homeodomain protein is usually not enough to recognize only its desired target genes. Most of the time, homeodomain proteins act in the promoter region of their target genes as complexes with other transcription factors.

3.2.1.1. Nkx2.5. Nkx2.5 is a homeodomain-containing transcription factor critical for cardiac development in species ranging from Drosophila to humans [80,81]. Heterozygous mutations of Nkx2.5 in humans cause congenital heart disease [82]. Mutations that affect DNA binding are associated with cardiac conduction defects or heart block. Nkx2.5 mRNA and protein are transiently upregulated during the formation of cardiac conduction system relative to the surrounding myocardium in embryonic chick, mouse, and human hearts, suggesting a role in the development of the conduction system [83].

Potential Nkx2.5-binding sites have been identified in the proximal promoters P1 of mouse/rat Cx40 and rat Cx43 genes. In fact, Linhares et al. [51] demonstrated that Nkx2.5 can interact specifically with the potential binding site present in the minimal promoter region of the mouse Cx40.

From the multiple experimental results obtained for mice expressing wild-type and a mutant Nkx2.5 and for Nkx2.5 homozygous or heterozygous knockout mice, we may conclude that Nkx2.5 can act as an activator as well as repressor of Cx43. Kasahara et al. [84a] reported that expression of Cx40 and Cx43 was dramatically decreased in the transgenic heart expressing a DNA binding-impaired mutant of mouse Nkx2.5, suggesting a positive role of Nkx2.5 in Cx40 and Cx43 gene regulation. A subsequent study by Kasahara et al. [84b] demonstrated that cardiomyocytes expressing wild-type Nkx2.5 or a putative transcriptionally active mutant (carboxyl-terminus deletion mutant) had dramatically reduced expression of Cx43, and that Cx43 was downregulated in wild-type Nkx2.5 adenovirus-infected adult cardiomyocytes as early as 16 h after infection, indicating that Cx43 downregulation is due to Nkx2.5 overexpression but not due to heart failure phenotype in vivo. Teunissen et al. [56] also reported that Cx43 protein and mRNA in rat primary neonatal

ventricular cardiomyocytes were significantly decreased after infection with adenovirus encoding Nkx2.5, and that the rat Cx43 proximal promoter P1 was down-regulated approximately twofold upon Nkx2.5 overexpression. These results showed that Nkx2.5 can act as an activator as well as repressor of Cx43.

Jay et al. [85] found that an entire population of Cx40 (−)/Cx45 (+) cells is missing in the atrioventricular node of Nkx2.5 heterozygous knockout mice but that, surprisingly, the cellular expression of Cx40, the major gap junction isoform of Purkinje fibers and a putative Nkx2.5 target, is unaffected. These data demonstrate that half-normal gene dosage of Nkx2.5 is sufficient for specific expression of Cx40 in the conduction system.

Dupays et al. [86] investigated cardiac connexin expression in the cardiovascular systems of wild-type and Nkx2.5-knockout 9.2 days post-conception (dpc) mouse embryos and found that the disruption of the Nkx2.5 gene in the mouse heart results in the loss of Cx43 (due in part to the poor development of the ventricular trabecular network) and downregulation of Cx45 gene expression. These results indicate that Nkx2.5 is involved in the transcriptional regulation of the Cx45 gene expression. Concerning Nkx2.5 haploinsufficiency, they showed that RNA extracts from wild-type and heterozygous mutant embryos expressed the Cx45, −43, and −40 genes, and that there were no marked differences between wild-type and heterozygous mutant embryos, suggesting that half-normal gene dosage of Nkx2.5 is sufficient for expression of Cx45, Cx43, and Cx40 in the hearts of 9.2 dpc mouse embryos.

Pashmforoush et al. [87] generated mice with a ventricular-restricted knockout of Nkx2.5, which display no structural defects but have progressive complete heart block, and massive trabecular muscle overgrowth found in some patients with Nkx2.5 mutations. They found that Cx40 was virtually absent in the adult mutant conduction system, while present in the neonatal mice. These data were consistent with Nkx2.5 being a positive regulator of Cx40 gene expression and further suggest a specific role for Nkx2.5 in the postnatal conduction system maturation and maintenance.

3.2.1.2. Shox2. Shox2 encodes a member of a small subfamily of paired, related homeodomain transcription factors that has been identified by virtue of its sequence similarity to the short-stature homeobox gene SHOX, causing various short-stature syndromes.

Blaschke et al. [88] generated Shox2 knockout mice. Homozygous mutant embryos were embryonic lethal at 11.5 to 13.5 dpc and exhibited severe hypoplasia of the sinus venosus myocardium. They showed aberrant expression of Cx40, Cx43, and Nkx2.5 within the SAN region. Similarly, Espinoza-Lewis et al. [89] reported that Shox2 is restrictedly expressed in the sinus venosus region including the SAN and the sinus valves during embryonic mouse heart development. Shox2 null mutation resulted in embryonic lethality due to cardiovascular defects, including bradycardia and severely hypoplastic SAN and sinus valves. Cx40 together with Nkx2.5 and natriuretic precursor peptide A (*Nppa*) was ectopically activated in the mutant SAN, where Tbx3 expression was lost. As described below, Tbx3 represses Cx40 expression by directly binding to the promoter of Cx40 gene. Thus, it has been suggested that Shox2 operates upstream of Nkx2.5, Tbx3, Cx40, and Cx43 to regulate the SAN genetic program.

3.2.1.3. Homeodomain-only protein (Hop). Hop encodes a 73 amino acid protein that contains a domain (the 60 amino acid homeodomain) homologous to those seen in homeobox transcription factors. Unlike all other known homeobox transcription factors, Hop does not directly bind DNA. It is expressed in the embryonic heart and plays an important role in development of the adult cardiac conduction system.

Ismat et al. [90] generated Hop knockout mice and found significant reduction in Cx40, but not Cx43, mRNA expression in hearts from homozygous knockout mice. Although in Hop heterozygous knockout mice, Cx40 protein is expressed throughout the cardiac conduction system, Cx40 expression is markedly downregulated in the region of the AVN and proximal cardiac conduction system in homozygous knockout mice. A subsequent study by Liu et al. [91] described that in transgenic mice overexpressing Hop, atrial Cx40 expression was reduced in left ventricular hypertrophy and normalized by TSA, whereas Cx43 expression was not changed. These results indicate that adequate amount of Hop expression is required for proper expression and localization of Cx40 in the cardiac conduction system.

3.2.1.4. Iroquois homeobox gene 3 (Irx3). The Iroquois homeobox (Irx) gene family of transcription factors contains a highly conserved DNA-binding homeodomain of the 3-amino acid loop extension superclass and is characterized by an 11-amino acid Iro motif. Irx genes have evolutionarily conserved roles during embryonic development [92] and can act as either repressors or activators of gene expression depending on the cellular context. All six Irx genes are expressed in partially overlapping patterns in the developing mouse heart [93,94].

Zhang et al. [95] very recently showed that Irx3 antithetically regulates Cx43 and Cx40 in the cardiac His–Purkinje network: Irx3 directly represses Cx43 transcription and indirectly activates Cx40 transcription. Promoter analysis of cardiac connexins (Cx40, Cx43, Cx45, and Cx30.2) revealed that the Cx43 promoter contains an evolutionarily conserved element harboring a putative Irx3 binding site, which overlaps with an Nkx2.5 binding motif (Irx/NKE) immediately upstream of conserved T-box binding elements. Coimmunoprecipitation analysis showed that Irx3 can form a protein complex with Nkx2.5. They found that Irx3 indeed antagonizes Nkx2.5-dependent activation of a Cx43-luciferase reporter containing 1.68 kb of the endogenous promoter sequence in transfected COS7 cells. Furthermore, three point mutations, made to alter predicted core binding sequences recognized by Irx3, diminished the ability of Irx3 to exert repression of Cx43-luciferase in the presence of increasing amounts of Nkx2.5. These results indicate that in the proximal VCS, where these transcription factors at expressed at high levels, Irx3 could therefore repress Nkx2.5-mediated activation of Cx43 transcription.

3.2.2. T-box transcription factors (Tbx5, Tbx2, Tbx3, Tbx18)

T-box transcription factors containing the T-box, which possesses a helix-loop-helix-type DNA-binding domain, form a large family that plays a crucial role in several developmental processes [96–98]. The T-box, however, is not only a conserved DNA-binding region, but also a conserved interaction domain for other transcription factors, chromatin remodeling complexes and histone-modifying enzymes involved in transcriptional control [79]. In mouse and man, 17 Tbx genes organized in five subfamilies are present. This family constitutes activators (e.g., Tbx5) and repressors (e.g., Tbx2 and Tbx3) of transcription that recognize the same binding element.

3.2.2.1. Tbx5. Tbx5 is one member of a transcription factor family containing the T-box. Tbx5 has been implicated in vertebrate tissue patterning and differentiation [96]. A role for Tbx5 in cardiac morphogenesis has been implied from studies of Holt–Oram syndrome, a rare autosomal dominant human disease caused by Tbx5 mutations [99–101]. Holt–Oram syndrome patients invariably exhibit upper limb malformations and have high incidences of both congenital heart disease, such as tetralogy of Fallot or hypoplastic left heart syndrome [102–104], and abnormal cardiac electrophysiology (particularly atrioventricular block). Tbx5 has a unique pattern of expression; transcripts are abundant in the posterior regions of the embryonic heart and predominate in the myocardium of the left atria, right atria, left ventricle, inflow tract (IFT), atrioventricular canal (AVC), inner curvature, and the atrial septum. Tbx5 is also expressed in the central conduction system (CCS), comprising the SA node, atrioventricular junction including the AV node, and the interventricular (IV) ring including its derivatives, the retroaortic root branch, right atrioventricular ring bundle, atrioventricular bundle (AVB), and

proximal part of the BBs. However, expression of Tbx5 is virtually absent from the right ventricle and outflow tract (OFT) [104–106].

Potential T-box binding sites have been identified in rat and mouse Cx40 promoters. Bruneau et al. [107] found five potential T-box binding sites in 1-kb fragments of mouse and rat Cx40 promoters and showed that Tbx5 binds to at least two of those sites. Linhares et al. [51] also demonstrated binding of Tbx5 to the putative T-box binding site with the mouse proximal Cx40 promoter (150 bps upstream of the transcription start site).

A crucial role of Tbx5 in regulation of Cx40 expression has been revealed by Tbx5 heterozygous knockout (haploinsufficient) mice, a model of human Holt–Oram syndrome. Bruneau et al. [107] found that Tbx5 haploinsufficiency markedly decreased Cx40 mRNA transcription in the heart, indicating that Cx40 is a Tbx5 target gene and that half-normal gene dosage of Tbx5 is insufficient for expression of Cx40. They also showed that when the mouse Cx40 promoter contained within a 1010-bp fragment joined with reporter luciferase construct was cotransfected with RSV-promoted Tbx5 cDNA into noncardiac cells (CV-1), Cx40 promoter activity increased more than 20-fold, demonstrating that the Cx40 promoter is a direct Tbx5 target. Furthermore, they observed synergistic activation of the mouse Cx40 promoter by Tbx5 and Nkx2.5.

In contrast to Nkx2.5, overexpression of Tbx5 has not been reported to downregulate connexin expression. Using clones of the P19Cl6 embryonal carcinoma cell line stably transfected with Tbx5, Fijnvandraat et al. [108a] found a strong positive correlation between the levels of Tbx5 transgene mRNA and of Cx40 mRNA, indicating that Tbx5 does not function as a repressor of connexin expression.

Development of ventricular conduction system including Cx40 expression requires cooperation between Tbx5 and Nkx2.5. Moskowitz et al. [108b] found that Cx40 expression is absent from the region of the AV bundle and bundle branches of $Tbx5^{+/-}$/$Nkx2.5^{+/-}$ mice, and these compound heterozygous mice exhibit ventricular conduction system delays.

3.2.2.2. Tbx2 and Tbx3. Among the T-box transcription factors, Tbx2 and Tbx3 function as repressors of transcription, including that of Cx40 and Cx43 expression. Christoffels et al. [109] showed that when the putative regulatory sequences of mouse Cx40 (a 1.2-kb mouse Cx40 upstream region from −1,196 to +62 relative to the transcription start site) and rat Cx43 (a 1.6-kb rat Cx43 upstream region from −1,338 to +281) were coupled to the luciferase reporter gene and transfected to Cos-7 or HEK cells, Cx40 promoter activity and to a lesser extent Cx43 promoter activity were repressed by Tbx2. They also found that at mouse developmental stages (embryonic days 9.5–14.5), expression patterns of Tbx2 and cardiac chamber-specific genes including Cx40 and Cx43 were mutually exclusive in the myocardium. Tbx2 is expressed in the IFT, AVC, inner curvature, and OFT, whereas no Tbx2 expression could be observed in the atrial and ventricular chamber myocardium. On the other hand, Cx40 and Cx43 are expressed in the atrial and ventricular chamber myocardium, but absent in the IFT, AVC, inner curvature, and OFT. These data indicate a role of Tbx2 in the repression of the connexin genes in hearts in vivo.

Hoogaars et al. [106] showed that, similarly to Tbx2, the patterns of Tbx3 and cardiac chamber-specific genes including Cx40 and Cx43 are mutually exclusive throughout all stages of mouse heart development (E9-E17.5). In the formed heart, Tbx3 is expressed in the central conduction system, comprising the SAN, AVN, bundle and proximal BBs, as well as the internodal regions and the atrioventricular region. Expression of Cx40 and Cx43 is specifically absent from the Tbx3 expression domain. However, there are some exceptions: at E15.5, weak Tbx3 expression protrudes further into the proximal BB which by then also expresses Cx40, and at E17.5, when the heart has reached its mature form, the AVB expresses low levels of Cx40, coexpressed with Tbx3. Hoogaars et al. [106] also demonstrated that Tbx3 dose-dependently represses the activity of mouse Cx40 promoter contained in 1.2-kbp Cx40 upstream regulatory region −1196 to +62 relative to the transcription start site. A subsequent study using ectopic expression of Tbx3 in mice done by Hoogaars et al. [110] revealed that Tbx3 represses the atrial phenotype including Cx43 and Cx40 expression and imposes the SAN phenotype including Cx30.2 expression on the atria. The mice displayed arrhythmias and developed functional ectopic pacemakers. Chromatin immunoprecipitation showed that Tbx3 specifically interacts with the TBE-containing DNA region of the Cx43 promoter, indicating that Tbx3 directly represses Cx43. These data identify a Tbx3-dependent pathway for the specification and formation of the SAN, and show that Tbx3 regulates the pacemaker gene expression program and phenotype.

Concerning molecular mechanisms by which Tbx2 and Tbx3 repress Cx43 expression, Boogerd et al. [111] demonstrated that muscle segment homeobox genes Msx1 and Msx2 together with Tbx2 and Tbx3 suppress Cx43 promoter activity and down regulate Cx43 gene activity in a rat heart-derived cell line. Using chromatin immunoprecipitation analysis they demonstrated that Msx1 can bind the Cx43 promoter at a conserved binding site located in close proximity to a previously defined T-box binding site, and that the activity of Msx proteins on this promoter appears dependent in the presence of Tbx3. These data indicate that Msx1 and Msx2 can function in concert with the T-box proteins to suppress Cx43 and other working myocardial genes.

It should be noted that expression of Cx45 in the heart seems to be regulated independently of repressive function of Tbx2 and Tbx3, because in the adult myocardium, Cx45 is expressed in all components of the conduction system including the Tbx3-positive central conduction system and the Tbx3-negative peripheral conduction system encompassing the distal part of the BBs plus the peripheral ventricular conduction network (the Purkinje fibers).

Apart from regulation of connexin expression in the heart, Chen et al. [112] reported that in osteoblast-like cells, the mouse Cx43 promoter (−479 to 0) contains two Tbx2 binding sites. This binding was dependent on the TCACAC consensus sequence, and transient transfection analysis with a Cx43 promoter-driven lacZ reporter construct revealed negative regulation of Cx43 transcription mediated by these two Tbx2 binding sites.

3.2.2.3. Tbx18. It has been shown that Tbx18-expressing mesenchymal precursor cells give rise to the SAN, especially the head region, which represents ~75% of the SAN volume and originates pacemaker activity in the early embryonic heart [113]. In contrast, the surrounding right atrium is negative for Tbx18 and originates from Isl1-expressing second heart field mesodermal progenitors [114].

Kapoor et al. [115] recently demonstrated that Tbx18 specifically down-regulates Cx43 at both the mRNA and protein levels, while having no effect on Cx40 or Cx45. Additionally, Cx43 promoter activity is directly repressed by Tbx18 and Tbx3, but not by Tbx20, which plays a role during ventricular chamber formation.

3.2.3. GATA family

GATA transcription factors contain a highly conserved DNA binding domain consisting of two zinc fingers of the motif Cys-X2-Cys-X17-Cys-X2-Cys that directs binding to the nucleotide sequence element (A/T)GATA(A/G) [116]. Based on their expression patterns, the GATA proteins have been divided into two subfamilies, GATA-1, -2, and -3 and GATA-4, -5, and -6. GATA-1, -2, and -3 genes are prominently expressed in hematopoietic stem cells, where they regulate differentiation-specific gene expression in T-lymphocytes, erythroid cells, and megakaryocytes. GATA-4, -5, and -6 genes are expressed in various mesoderm- and endoderm-derived tissues such as heart, liver, lung, gonad, and gut, where they play critical roles in regulating tissue-specific gene expression.

Linhares et al. [51] showed that GATA4 can interact specifically with its putative site (−49, –36) in the rat/mouse Cx40 proximal promoter, and that the coexpression of GATA4 induced an approximate six-fold increase in luciferase activity from −1190/+121Cx40Luc. Addition of Nkx2.5 with GATA4 led to a 10-fold increase in activation. The same effect was observed when the minimal promoter −50/+121 Cx40Luc construct was used, indicating that this region contains the sites required for activation of Cx40 expression by both Nkx2.5 and GATA4 transcription factors.

Munshi et al. [2] performed Cx30.2 (*Gjd3*) enhancer analysis and identified GATA4 as a regulator of atrioventricular delay via regulation of Cx30.2 expression. They found that A 660 bp Cx30.2 enhancer is sufficient to direct atrioventricular conduction system-specific gene expression, the Cx30.2 minimal enhancer harbors one GATA element and two T-box elements that direct DNA binding and ternary complex formation of Gata4 and Tbx5.

Fig. 1 summarizes a current view of regulation of cardiac connexin expression in relation to myocardial cell lineages.

3.2.4. HNF-1

HNF-1 is a transcription factor whose expression has been positively correlated with the differentiated hepatic phenotype and positively regulates many liver-specific genes, e.g., α1-antitrypsin, albumin, and α- and β-fibrinogen [117]. Two isoforms, HNF-1α and HNF-1β, are known; the former is highly expressed in adult liver, whereas the latter is expressed earlier in development. HNF-1 isomers dimerize in a homomeric or heteromeric manner before binding to DNA, and each combination can have distinct effects on transcriptional control, depending upon the target gene. HNF-1 is also expressed in other tissues such as kidney, gut, and pancreas, where it controls the expression of many genes.

Hennemann et al. [34] first reported that the promoter region of the mouse Cx32 gene contains two putative binding sites for HNF-1 within 680 bp upstream of the main transcription start site. Piechocki et al. [31] showed that promoter activity of the rat Cx32 promoter (nt −753 to −33) linked to the luciferase gene was correlated with the binding of HNF-1 to two HNF-1 consensus sites centered at −187 and −736, and that expression of HNF-1 and binding to these elements were only observed with rat hepatoma (MH1C1) cells that express endogenous Cx32. Koffler et al. [35] demonstrated that stable transfection of non-Cx32-expressing WB-F344 rat liver epithelial cells with HNF-1α stimulated a transfected Cx32 promoter element (mp −244 to −33), binding of HNF-1α to the −187 site, and expression of endogenous Cx32, and that Cx32 expression was also significantly decreased in HNF-1α (−/−) mice. These data suggest that HNF-1 determines the cell-specific expression of Cx32 based on the cell-type-independent activation of Cx32 promoter by ubiquitous transcription factors such as Sp1 as described above.

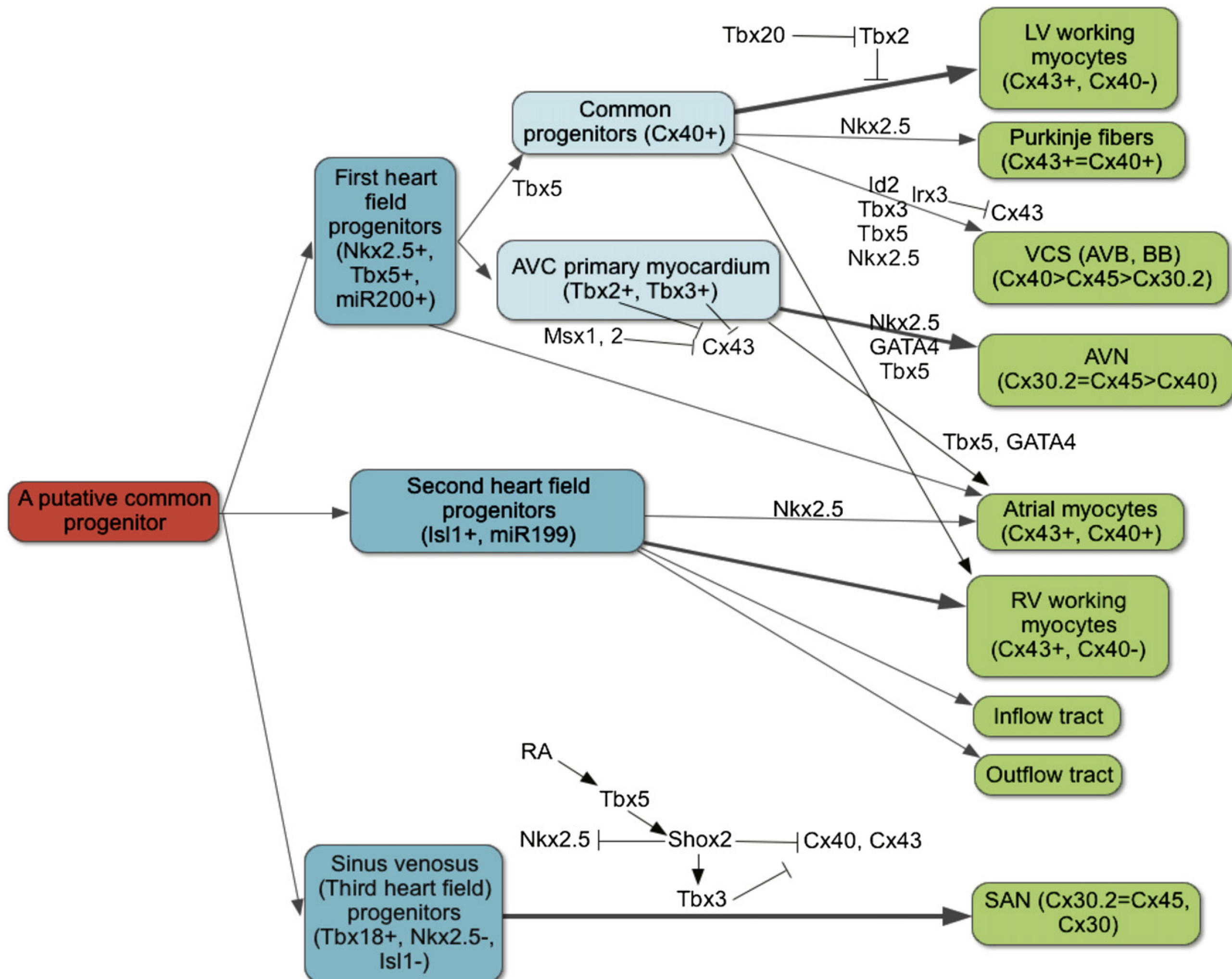

Fig. 1. A current view of regulation of cardiac connexin expression in relation to myocardial cell lineages. AVB, atrioventricular bundle; AVC, atrioventricular canal; AVN, atrioventricular node; BB, bundle branch; LV, left ventricle; RV, right ventricle; SAN, sinoatrial node; VCS, ventricular conduction system.

3.2.5. Mist1

Mist1 is one of the members of the basic helix-loop-helix (bHLH) protein family that are instrumental the in development of numerous organ systems. Rukstalis et al. [118] found that Mist1 knockout (KO) pancreatic acini are deficient in gap-junctional intercellular communication, mainly due to the loss of Cx32 expression, and that Cx32 mRNA and protein levels are greatly reduced in the Mist1 KO exocrine pancreas, whereas Mist1 KO acinar cells continue to express the Cx26 gene. They also showed that co-transfection of cells with the mouse *Cx32* P1 promoter (−680 to +20) -luciferase reporter plasmid (*Cx32*p-Luc) and an expression plasmid encoding *Mist1* generated a 15- to 20-fold increase in *Cx32*p-Luc expression, whereas no other bHLH transcription factor such as E12, E47, HEB, PTF1-p48, NeuroD, MyoD, and Mash1, could activate expression of the *Cx32*p-Luc gene. The ability of Mist1 to activate expression of the *Cx32*p-Luc gene was dependent on its DNA binding and dimerization properties. From those results, it is concluded that an active Mist1 protein is required to generate full *Cx32* gene expression in secretory acinar cells.

Concerning tissue-specific Cx32 expression, combined data on Mist1 [118] and HNF-1 indicate that there are at least two different activation mechanisms of Cx32 expression in the epithelial cell: Mist1-dependent expression in secretory exocrine cells, including the pancreas, submandibular gland, parotid gland and seminal vesicles and HNF-1-dependent expression in the hepatocyte. Rukstalis et al. [118] described that Cx32 and Mist1 co-expression is observed in all secretory exocrine cells, including the pancreas, submandibular gland, parotid gland and seminal vesicles, whereas hepatocytes express Cx32 but not Mist1. Furthermore, they showed that in the liver tissue of Mist1 KO mice, expression of *Cx32* transcripts and formation of Cx32-containing gap junction plaques remained unchanged, indicating that Cx32 expression in the liver is independent of Mist1. On the other hand, Koffler et al. [35] reported that Cx32 mRNA content in HNF-1α homozygous KO mouse liver was approximately one-third that of HNF-1a heterozygous KO mice, suggesting a crucial effect of HNF-1 on Cx32 in the hepatocyte.

3.2.6. Sox10 and early growth response gene-2 (Egr2/Knox20)

The Sox proteins belong to the high mobility group (HMG) box superfamily of DNA-binding proteins. Among them, Sox10 is first expressed widely in cells of the neural crest at the time of their emergence, and later in neural crest cells that contribute to the melanocyte lineage and to the forming peripheral nervous system. Its expression is detected in the enteric, sensory, and sympathetic ganglia as well as along nerves in a manner typical for the Schwann cell lineage [119].

The early growth response gene-2 (Egr2/Knox20) gene was originally identified as a serum response immediate-early gene that encodes a protein with three tandem zinc fingers of cys(2)-his(2) class [120–122]. Egr2/Krox20 knockout mice showed disrupted hindbrain segmentation and development and a block of Schwann cells at an early stage of differentiation [123,124].

Mutations in Sox10 genes are discovered in patients with various hereditary neurological diseases including Waardenburg–Hirschsprung disease or Shah-Waardenburg syndrome (WS4), and WS4 with peripheral neuropathy consistent with Charcot–Marie–Tooth disease type I [119]. Mutations in Egr2/Krox20 gene are identified in patients with congenital hypomyelinating neuropathy and a family with Charcot–Marie–Tooth disease type 1D (CMT1) [124].

Bondurand et al. [36] showed that Sox10, in synergy with Egr2/Knox20, strongly activates expression of Cx32, a major protein of peripheral myelin, in vitro by directly binding to its nerve-specific promoter P2, and that Sox10 and Egr2 mutants identified in patients with peripheral myelin defects failed to transactivate the Cx32 promoter P2. They also demonstrated that a T-to-G point mutation at position −528 of the Cx32 promoter P2 identified in some CMTX1 patients eliminates binding and activation by Sox10. Similarly, Houlden et al. [37] identified a large family with Charcot–Marie–Tooth disease with a G-to-C point mutation at position −526 bp of the Cx32 promoter P2 and showed that this mutation reduced the activity of the Cx32 promoter and the affinity for Sox10 binding. These data suggest that interaction between the Cx32 P2 promoter, Sox10, and Egr2 plays an important role in Cx32 expression in the Schwann cell.

In addition to Cx32, Jungbluth et al. [125] reported that segment-specific expression of Cx31 in the mouse embryonic hindbrain is positively regulated by Egr-2/Krox20. They found that the spatiotemporal patterns of Cx31 expression in rhombomeres 3 and 5 and in the boundary cap cells are very similar to the expression pattern of Egr2/Krox20, and that in Egr2/Krox20 KO embryos, no Cx31 expression was detectable in the developing hindbrain, whereas expression of Cx31 in other sites nonoverlapping with that of Egr2/Krox20, such as in the posterior end of the embryonic body axis, was not affected in Egr2/Krox20 KO embryos.

Furthermore, Schlierf et al. [126] showed that Sox10 regulates not only Cx32 expression but also Cx47 (Gjc2) expression in oligodendrocytes. Osaka et al. [127] identified a homozygous mutation (c.-167A>G) within the Cx47 (*GJC2*) promoter at a potent SOX10 binding site in a patient with mild Pelizaeus–Merzbacher-like disease (PMLD). Functionally, this mutation completely abolishes the SOX10 binding and attenuated mouse Cx47 promoter activity in human glioblastoma U138 cells. Combes et al. also found this mutation in 7 PMLD patients. However, their functional analysis of the c.-167A>G mutation on the human Cx47 promoter showed a higher activity with mutated promoter than with the wild type in COS-7 and HEK293 cells. Thus, the functional consequences of this mutation remain to be fully elucidated.

3.2.7. Estrogen and progesterone

Cx43 expressed in the uterus is thought to play a critical role in the onset of labor by allowing for an increase in myometrial cell coupling and coordinated synchronous contraction of the muscle at the end of pregnancy. Before the onset of labor, there is a dramatic increase in both mRNA and protein levels of Cx43 in the myometrium [128,129]. The Cx43 gene in the myometrium is under the control of steroid hormones, being up-regulated by estrogen and down-regulated by progesterone (PR) [130]. Yu et al. [16] showed that the rat Cx43 promoter contains several sequences resembling half the palindromic estrogen response elements (half-EREs); depending on the concentration of estrogen, the half-EREs were functional when cotransfected with estrogen receptor cDNA into HeLa cells.

However, Oltra et al. [131] described that these half-EREs are not required for the induction of the Cx43 gene; a promoter containing only 145 nucleotides and lacking all half-EREs was shown to be sufficient to achieve full estrogen response. It has also been reported that elevated Cx43 expression in term human myometrium is independent of myometrial estrogen receptors [58]. On the other hand, it has been shown that the expression of several AP-1 family transcription factors (c-jun and c-fos family of transcription activators) is dramatically increased in the myometrium before the onset of labor and that this expression is regulated by both mechanical and hormonal stimuli [17,58,64]. Therefore, AP-1 proteins play crucial roles in positive regulation of Cx43 expression in the pregnant myometrium as described above.

PG signaling plays important roles in the maintenance of pregnancy by repressing the expression of labor-associated proteins including Cx43. Dong et al. [132] showed that PR represses transcription of Cx43 gene, an effect dependent on the presence of AP-1 site within the proximal Cx43 promoter. p54nrb (non-POU-domain-containing, octamer binding protein), can function as a PR corepressor and interacts directly with PR independent of progesterone. Mutation of AP-1 site abolishes PR-mediated repression and decreases the recruitment of PR and p54nrb onto the Gja1 promoter. Furthermore, knockdown p54nrb expression by small interfering RNA alleviates PR-mediated repression on Cx43 transcription, whereas overexpression of p54nrb enhances it. In the physiological context of pregnancy, p54nrb protein levels

decrease with the approach of labor in the rat myometrium. These data indicate that decreased expression of p54nrb at the time of labor may act to derepress PR-mediated inhibition on Cx43 expression and contribute to the initiation of labor.

Besides AP-1 proteins, Oltra et al. [131] reported that Ini, a small nuclear zinc-finger protein, actively participates in the positive response of Cx43 expression to estrogen. They showed that Ini binds to the rat proximal Cx43 promoter between −71 and −34, its expression is ubiquitous, and in the uterus is upregulated by estrogen. Furthermore, transient transfection experiments performed with estrogen receptor alpha cDNA show that overexpression of Ini enhances, in a dose-dependent fashion, the up-regulation of the Cx43 gene by estrogen.

3.2.8. Thyroid hormone and parathyroid hormone

Stock and Sies [133] demonstrated that after treatment with thyroid hormone Cx43 mRNA was elevated 2.1-fold in rat liver samples as compared to controls, while there was no change in the heart. They identified thyroid hormone response elements in the rat Cx43 promoter region at position −480 to −464 and showed that the Cx-480 element formed stronger complexes with thyroid hormone receptor alpha/retinoid X receptor alpha heterodimers than with vitamin D receptor/retinoid X receptor alpha heterodimers. In transfected Cos-7 cells, promoter activation via this element was observed after treatment with 3,3′,5-triiodo-L-thyronine. de Montgolfier et al. [71] reported that Cx43 is expressed in the Sertoli cells of rainbow trout and that triiodothyronine (T3) regulates testicular Cx43 expression in brook trout testis. Single thyroid hormone (tr-beta) (−112 to −107) response elements are identified and electrophoretic mobility shift assays indicate the presence of competitive protein binding sites. Sequential deletion and point mutations in the tr-beta response element indicate that T3 stimulates Cx43 expression via direct regulation of gene transcription.

Mitchell et al. [134] showed that in the rat osteosarcoma cell line, parathyroid hormone (PTH) induced a 4-fold increase in activity of Cx43 promoter containing 1.6 kb 5′ of the transcription start site, in which responsive sequence was localized to between −31 and +1 bp. They demonstrated that PTH treatment of transgenic mice containing the 1.6 kb promoter luciferase construct induced increases in luciferase and Cx43 immunoreactivity in bone cells underlying the tibial growth plate. They also found that the full Cx43 3′UTR conferred a 3-fold response to PTH when placed 3′ of a CMV-luciferase construct, in which responsive sequence was localized to between 2510 and 3132 of the 3′-UTR. These data indicate that PTH responsive sequences are present in the Cx43 promoter and 3′-UTR, suggesting that transcriptional and posttranscriptional pathways operate to regulate PTH-induced Cx43 expression in osteoblast cells.

3.2.9. Other transcription regulators of connexin expression

Martin et al. [135] reported that the transcriptional repressor neuron-restrictive silencer factor (NRSF) controls neuron- and beta-cell-specific expression of Cx36 expression. They identified a putative neuron-restrictive silencer element conserved between rodent and human species in a 2043-bp fragment of the human Cx36 promoter, and showed that NRSF binds the neuron-restrictive silencer element. They also demonstrated that this factor is not expressed in insulin-secreting cells and neurons; viral gene transfer of NRSF in insulin-secreting beta-cell lines induced a marked reduction in Cx36 mRNA; repression of Cx36 expression by NRSF is mediated through the recruitment of histone deacetylase to the promoter of neuronal genes.

Ciliary neurotrophic factor (CNTF) is a member of the IL-6 family that is produced as a nonsecreted cytosolic cytokine by astrocytes within the central nervous system and has its specific receptor, termed ciliary neurotrophic factor receptor alpha (CNTFRalpha), located on neuronal cell membranes. Ozog et al. [136] demonstrated that the heterodimer CNTF–CNTFRalpha significantly increased Cx43 mRNA in normal astrocytes in a Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT)-dependent manner, whereas CNTF–CNTFRalpha did not alter Cx30 mRNA levels. In the promoter P1 region of mouse Cx43, they identified three putative CNTF-response elements (binding sites for STAT3 dimers that contain base sequences TTCCN$_{3-5}$AA) and showed that these three elements, located at regions −1510, −1179, and −893, are essential for *Cx43*-regulated expression by CNTF–CNTFRalpha.

4. Epigenetic regulation of connexin expression

In recent years, it has become clear that epigenetic processes, such as histone modifications, DNA methylation, and microRNA species, are also essentially involved in connexin gene regulation [8].

4.1. Histone modification

The involvement of histone modifications, and histone acetylation in particular, in the control of connexin expression has been demonstrated by works with histone deacetylase inhibitor (HDACi) [8]. Very few studies have actually addressed the molecular mechanisms that underlie the effects of HDACi on connexin expression.

Ogawa et al. [137] demonstrated that treatment of nonmalignant human peritoneal mesothelial cells with a HDACi, suberoylanilide hydroxamic acid (SAHA), at nanomolar concentrations caused a dose-dependent increase of Cx43 mRNA and protein expression, accompanied with the accumulation of acetylated histones H3 and H4 in the chromatin fragments associated with Cx43 gene. From these results, they discussed that SAHA-induced Cx43 gene expression could be ascribed to histone H3/H4 acetylation.

Hernandez et al. [60] found that the TSA-mediated induction of Cx43 in human prostate cancer cells depends on the recruitment of p300/CREB-binding protein, a transcriptional coactivator displaying histone acetyltransferase (HAT) activity, and the transcription factors AP-1 and Sp1 to the Cx43 gene promoter. This was accompanied by hyperacetylation of histones H4 surrounding the AP-1- and Sp1-responsive gene elements.

Hohl and Thiel [138] reported that Cx36 gene expression was enhanced by TSA 3.5–4x in pancreatic α (αTC1-9) and β (βTC3) cells, but TSA was unable to trigger Cx36 transcription in neuronal SN56 or pituitary AtT20 cells, indicating that cell type (pancreas)-specific determinants are important for TSA to have an impact of the transcription of the Cx36 gene. They found that this cell type-specific enhancement coincident with the state of epigenetic markers for active or silenced genes: methylation of lysine residue 9 (Lys9) of histone H3 functions as an epigenetic marker for silenced genes, whereas methylation of lysine residue 4 (Lys4) of histone H3 does as that for actively transcribed genes. Chromatin immunoprecipitation experiments using antibodies directed against methylated histone H3Lys4 or H3Lys9 showed that Cx36 gene is activated in βTC3 cell but silenced in AtT20 cells.

Zupkovitz et al. [139] found that in mouse embryonic stem cells, expression levels of Cx43 mRNA are reduced not only upon the loss of HDAC1 but also in a dose-dependent manner by TSA. Chromatin immunoprecipitation experiments demonstrated that HDAC1 is present at the Cx43 promoter in wild-type cells, while HDAC2 is recruited mainly in HDAC1-null cells. Analysis of chromatin modifications at the Cx43 promoter revealed that the acetylations of histone H4 and Lys9 at histone H3 are slightly reduced in the absence of HDAC1, while the trimethylation of Lys9 at histone H3 is increased in HDAC1-null cells. Thus, the reduced expression of Cx43 correlates with the increased presence of the epigenetic marker for silenced genes.

It also should be noted that HDACi's hyperacetylate nonhistones such as cytosolic proteins, including transcription factors themselves. Therefore, it remains to be clarified whether reported effects of HDACi's on connexin expression really result from histone modification. In

fact, Colussi et al. [140] showed that in the mouse dystrophic (mdx) heart, a prolonged treatment (2–3 mo) with HDACi SAHA prevents ventricular arrhythmias, reverses conduction defects, and recovers to a normal Cx43 distribution at intercalated disks from abnormal presence of lateralized Cx43 on mdx cardiomyocytes without significant changes in Cx43 protein expression. Subsequently, a work by Colussi et al. [141] provided evidence that, in mdx as well as normal hearts, Cx43 distribution in and out of intercalated disks and its function may be regulated by the degree of Nε-lysine acetylation. They found that global histone acetylase activity is abnormally elevated, the acetylase P300/CBP-associated factor (PCAF) coimmunoprecipitates with Cx43, which is Nε-lysine acetylated and lateralized in mdx heart, and that the level of acetylated Cx43 is significantly higher in mdx than in normal control hearts. In vivo treatment of mdx with the pan-histone acetylase inhibitor anacardic acid significantly reduced Cx43 Nε-lysine acetylation and restored its association to intercalated disks. On the contrary, a short-term treatment (96 h) of normal mice with SAHA, which increased total protein acetylation, determined dissociation of Cx43 from intercalated disks and lateralization. Thus, evidence is now accumulating about epigenetic processes meant for nonhistone protein modification including connexins.

4.2. Gene silencing by DNA methylation of the connexin promoters

The downregulation of connexin expression is often observed in tumors and transformed cell lines and is believed to contribute to the loss of proliferating control. Despite intensive studies, neither deletion nor mutation of connexin gene has been found common in human tumors. On the contrary, multiple studies have shown that epigenetic inactivation through hypermethylation of the promoter region could lead to silencing of connexin expression in various kinds of malignant cells, and that effects of DNA methyltransferase inhibitors on connexin expression depend on types of cells and connexins, as summarized in a review by Vinken et al. [8]. Among them, Chen et al. [142] reported that in human non-small cell lung cancers, lack of Cx43 mRNA expression in adjacent normal lung tissue was significantly correlated with micrometastasis into lymph nodes and that a higher frequency of promoter methylation was observed in Cx43 mRNA-negative patients compared with Cx43 mRNA-positive patients. Their data also indicate that promoter methylation may interfere with AP-1 binding to the promoter, resulting in lack of Cx43 gene expression. Sirnes et al. [143] very recently published a study of connexin expression and promoter methylation of connexin family members in normal colon, colorectal carcinomas, and colon cancer cell lines. They detected expression of six [Cx43 (GJA1), Cx59 (GJA9), Cx32 (GJB1), Cx26 (GJB2), Cx45 (GJC1), Cx31.9 (GJD3)] connexin genes in normal colonic tissue samples. Cx45 expression was reduced in colorectal carcinomas and cancer cell lines compared to normal tissue samples. A statistically significant association is found between the presence of promoter hypermethylation and reduced GJC1 expression. Cx45 was restored in cell lines treated with the demethylating drug 5-aza-2′deoxycytidine. Concerning other connexin genes than GJC1, the authors found that in colon cancer cell lines, the methylation status is not associated with gene expression.

4.3. MicroRNAs

Mature microRNAs (miRNAs) are ~22-nucleotide single-stranded non-protein-coding RNAs that inhibit the expression of specific mRNA targets through Watson–Crick base pairing between the miRNA 'seed region' and sequences commonly located in the 3 untranslated regions (UTRs). The human genome is estimated to encode up to 1000 miRNAs, which are either transcribed as standalone transcripts, frequently encoding several miRNAs, or generated by the processing of introns of protein-coding genes. The potential roles of miRNAs in controlling connexin expression have recently been investigated [8,144,145].

Kim et al. [146] demonstrated that the muscle-specific miRNAs, miR-206, miR-1 and miR-133, are induced during in vitro differentiation of C2C12 myoblast cells into multinucleate myotubes. Cx43 is shown to be a direct negative target for miR-206 by luciferase reporter assay. Anderson et al. [147] also showed that two related miRNAs, miR-206 and miR-1, inhibit the expression of Cx43 protein during myoblast differentiation in vitro and in vivo without altering Cx43 mRNA levels, and that Cx43 mRNA contains two binding sites for miR-206/miR-1 in its 3′-untranslated region, both of which are required for efficient downregulation

Yang et al. [148] showed that muscle-specific miRNA miR-1 is overexpressed in individuals with coronary artery disease, and that when overexpressed in normal or infarcted rat hearts, it exacerbates arrhythmogenesis. Elimination of miR-1 by an antisense inhibitor in infarcted rat hearts relieved arrhythmogenesis. miR-1 overexpression slowed conduction by post-transcriptionally repressing Cx43. The authors found that 3′-UTRs of Cx43 gene contain stretches of eight nucleotides that are complementary to the first eight nucleotides from the 5′ end of miR-1, and proved that miR-1 binds to these nucleotides. miR-1 is shown to reduce Cx43 levels also in isolated neonatal rat ventricular myocytes in culture. They discussed that miR-1 may have important pathophysiological functions in the heart, and that is a potential antiarrhythmic target.

Callis et al. [149] showed that miR-208a is required for proper expression of Cx40. Cx40 transcript and protein levels are markedly decreased in Mir208a −/− hearts compared with wild-type, whereas Cx40 transcript levels are not affected in miR-208a transgenic hearts. No readily apparent changes in Cx43 transcript levels are detected in either miR-208a Tg or Mir208a−/− hearts. These observations indicate that Cx40 is not directly regulated by miR-208a, but instead miR-208a targets a transcription factor associated with Cx40 and/or required for Cx40 expression. Furthermore, they found that the 3′ UTR of Gata4 mRNA contained a predicted miR-208a target site, that cotransfection of miR-208a with the luc-GATA4 reporter repressed luciferase activity, and that GATA4 protein levels were elevated in Mir208a−/− hearts compared with hearts from wild-type littermates. Consistent with posttranscriptional regulation, the GATA4 transcript levels are unchanged in Mir208a−/− hearts. Taken together, these observations demonstrate that miR-208a directly targets the cardiac transcription factor GATA4, suggesting that miR-208a regulates Cx40 expression possibly via GATA4. In addition to roles of miR-208a on Cx40 expression, Callis et al. [149] reported several novel findings: (a) miR-208a and miR-208b are members of miR-208 family, are encoded within introns of αMHC and βMHC, respectively, and are differentially expressed during heart development and heart pathology, paralleling the expression of their respective host genes, αMHC and βMHC. (b) Cardiac overexpression of miR-208a is sufficient to induce hypertrophy, which resulted in pronounced repression of the miR-208 regulatory targets thyroid hormone-associated protein 1 and myostatin, 2 negative regulators of muscle growth and hypertrophy. (c) miR-208a is necessary for normal cardiac conduction. miR-208a gain- and loss-of-function are associated with arrhythmias.

Inose et al. [150] revealed Cx43, a major gap junction protein in osteoblasts, as a target of miR-206 during osteoblast differentiation in vivo. They first showed that miR-206, previously viewed as a muscle-specific miRNA, is expressed in osteoblasts, its expression decreases over the course of osteoblast differentiation, and that overexpression of miR-206 in osteoblasts inhibits their differentiation, and conversely, knockdown of miR-206 expression promotes osteoblast differentiation. Next, they found two putative target sequences for miR-206 in the 3 UTR region of Cx43 and proved that ectopically expressed miR-206 binds these sites. Furthermore, they showed that ectopic expression of miR-206 downregulates endogenous

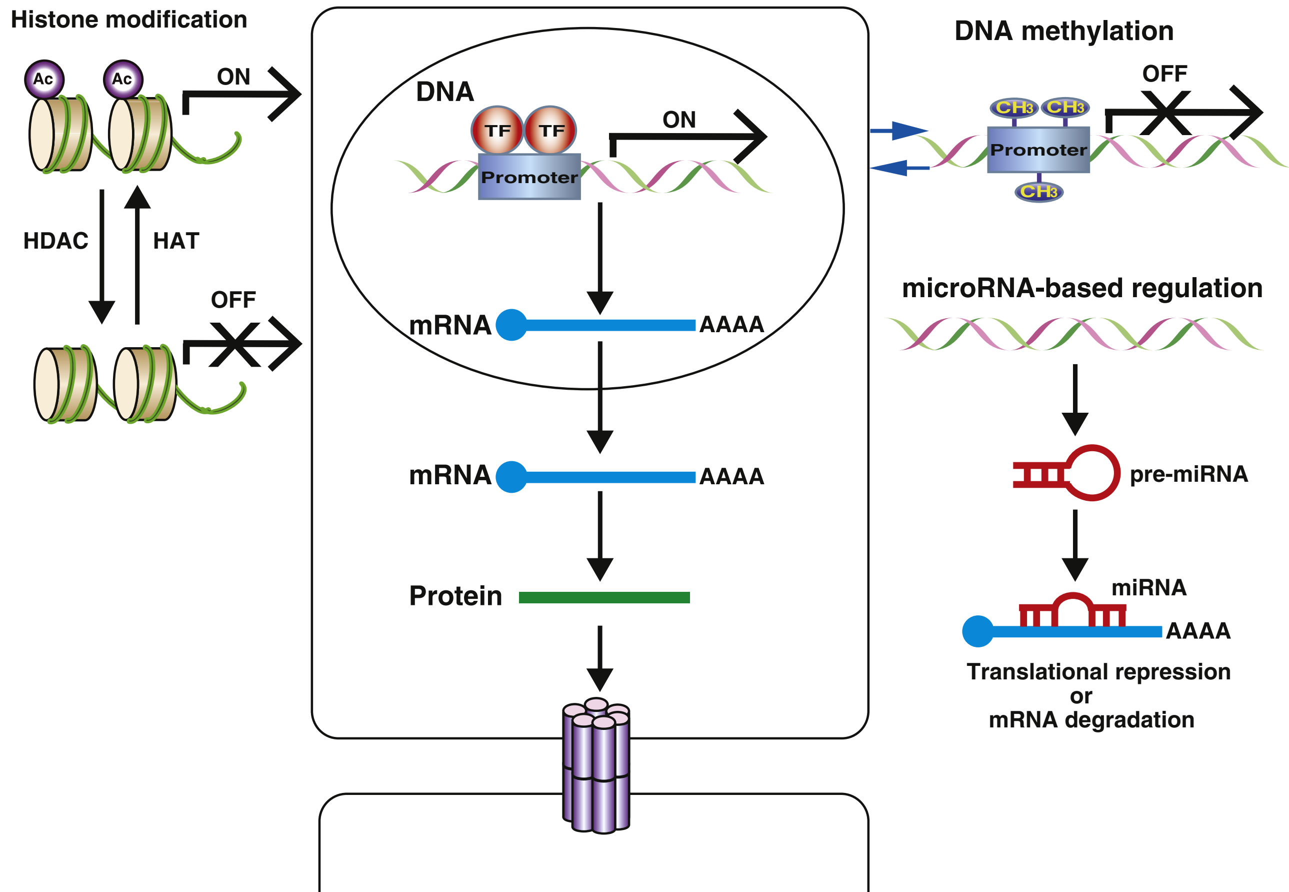

Fig. 2. Regulation of connexin expression by transcriptional factors and epigenetic mechanisms including histone modification, DNA methylation, and microRNAs. Ac, acetyl group; CH_3, methyl group; HAT, histone acetyltransferase; HDAC, histone deacetylase; miRNA, microRNA; pre-miRNA, precursor microRNA; TF, transcription factor.

Cx43 protein expression without affecting Cx43 mRNA expression and that continuous expression of miR-206 in osteoblasts decreases osteoblast differentiation and Cx43 protein expression. However, when they co-expressed Cx43 together with miR-206, the inhibitory effect of miR-206 on osteoblast differentiation is markedly rescued, indicating that restoration of Cx43 protein expression is sufficient to obtain normal osteoblast differentiation in miR-206-expressing osteoblasts.

5. Conclusion

Here, we have reviewed regulation of connexin expression by dividing into gene structure of connexins, transcriptional factors, and epigenetic regulation including histone modification, DNA methylation, and microRNAs (Fig. 2). As detailed above, it has become clear that in various biological processes, connexin transcription network is modulated by a combination of multiple transcription factors, histone modifications, and microRNAs. However, how the interaction between these regulatory mechanisms takes place is still an open question. Recently, Schlesinger et al. [151] demonstrated the validity of a system biology approach for the study of the cardiac transcription network, by integrating mRNA profiles with DNA-binding events of key cardiac transcription factors, activating histone modifications, and microRNA profiles obtained in wild-type and RNAi-mediated knockdown. Such an approach for the study of connexins may help us to further understand regulation of connexin gene expression under physiological and pathological circumstances and to develop potential connexin therapies for human diseases including cancer, heart, and neurological disorders.

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