Biol. Chem., Vol. 383, pp. 725 – 737, May 2002 · Copyright © by Walter de Gruyter · Berlin · New York

Review

Structural and Functional Diversity of Connexin Genes in the Mouse and Human Genome

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Gap junctions are clustered channels between contacting cells through which direct intercellular communication *via* diffusion of ions and metabolites can occur. Two hemichannels, each built up of six connexin protein subunits in the plasma membrane of adjacent cells, can dock to each other to form conduits between cells. We have recently screened mouse and human genomic data bases and have found 19 connexin (Cx) genes in the mouse genome and 20 connexin genes in the human genome. One mouse connexin gene and two human connexin genes do not appear to have orthologs in the other genome. With three exceptions, the characterized connexin genes comprise two exons whereby the complete reading frame is located on the second exon. Targeted ablation of eleven mouse connexin genes revealed basic insights into the functional diversity of the connexin gene family. In addition, the phenotypes of human genetic disorders caused by mutated connexin genes further complement our understanding of connexin functions in the human organism. In this review we compare currently identified connexin genes in both the mouse and human genome and discuss the functions of gap junctions deduced from targeted mouse mutants and human genetic disorders.

Key words: Alternative splicing / Connexin-deficient mice / Human connexin genes / Human connexin mutants / Mouse connexin genes.

Introduction

Gap junctions are used for direct intercellular communication by docking of two hemichannels in adjacent cells, thereby forming conduits between their cytoplasmic compartments. Gap junction channels allow diffusional exchange of ions, metabolites and second messengers (Figure 1A). Each hemichannel (connexon) is composed of six protein subunits, called connexins (Cx) (Figure 1B). Each connexin protein comprises four transmembrane, two extracellular and three cytoplasmic domains (*i.e*. N-terminus, cytoplasmic loop, and C-terminus, Figure 1C; *cf.* Kumar and Gilula, 1996). Interestingly, invertebrates express topologically and functionally similar channel forming proteins, designated as innexins, which, however, show no sequence homology to connexins (Phelan *et al.*, 2001).

So far 17 connexin genes have been described and characterized in the murine genome. For most of them, orthologs in the human genome have been found (see White and Paul, 1999; Manthey *et al.*, 1999; Teubner *et al.*, 2001; Söhl *et al.*, 2001b). The expression pattern of different connexin transcripts and proteins in different cell types and organs suggest that there is a widespread spatio-temporal overlap. Two connexons consisting of the same type of connexin proteins can form a homotypic channel, whereas two connexons each consisting of different connexins can form a heterotypic gap junction channel. A much larger variety would be generated if the connexin subunits differ within one connexon (heteromeric hemichannel) (*cf*. Figure 1D). The occurrence of certain heteromeric connexin channels has been demonstrated by co-immunoprecipitation after purification of connexons in the presence of detergents. Heterotypic or heteromeric connexin channels may have distinct permeabilities, which may differ from the corresponding homotypic and homomeric channels (Elfgang *et al.*, 1995). Almost all cells in the developing and adult mammalian organism are interconnected *via* gap junction channels, with very few exceptions, for example fully differentiated skeletal muscle cells, erythrocytes, platelets, and sperm. In order to find out how many different connexin genes exist in the mouse and human genome, we recently performed searches in murine and human genomic libraries available with the Celera company and at the EMBL/Heidelberg or NCBI (Eiberger *et al.*, 2001). Here we present an overview on distinct connexin genes present in the mouse and human genome, which may include all members of this gene family, provided that no further connexin gene will be discovered in the remaining non-sequenced portion (about 1 – 5%) of the genomes. Due to space limitations we cannot quote all relevant publica-

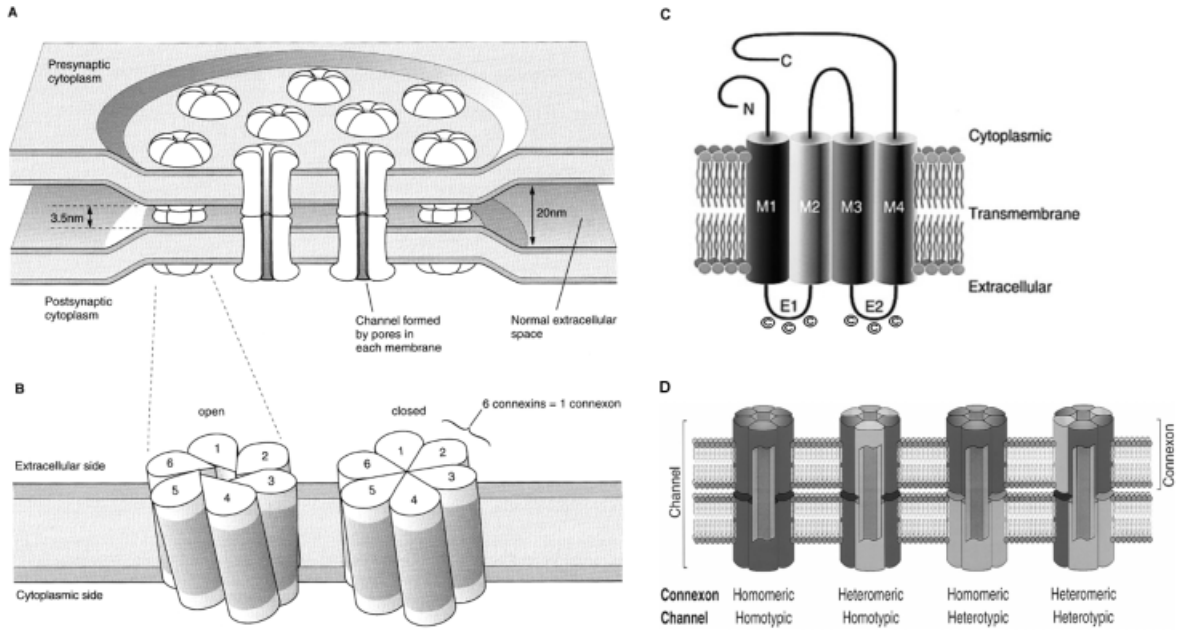

Fig. 1 Three-Dimensional Model of the Gap Junction Channel Based on X-Ray Diffraction Studies.
(A) Each apposed cell contributes a hemichannel to the complete gap junction channel. Each hemichannel, about 1.5 – 2.0 nm in diameter, is formed by six protein subunits called connexins. Each connexin is about 7.5 nm long, spanning the membrane and contacting two connexins in the hemichannel across the gap between the cells (Yeager *et al.*, 1998). At these sites the cells are only 3.5 nm apart (Markowski *et al.*, 1977) (adapted from Kandel *et al.*, 1995).
(B) Model of a single connexon. Six connexin subunits of the hemichannel may coordinately change configuration to open and close the hemichannel. Closure is achieved by the subunits sliding against each other and tilting at one end, thus rotating at the base in a clockwise direction. Each subunit is thought to move about 0.9 nm at the cytoplasmic surface. The darker shading indicates the portion of the connexon embedded in the membrane (Unwin and Zampighi, 1980) (adapted from Kandel *et al.*, 1995).
(C) Topological model of a connexin. The cylinders represent transmembrane domains (M1-M4). The loops between the first and the second, as well as the third and fourth transmembrane domains are predicted to be extracellular (E1 and E2, respectively), each with three conserved cysteine residues (adapted from Kumar and Gilula, 1996).
(D) Schematic drawing of possible arrangements of hemichannels to form complete gap junction channels. Hemichannels consisting of six connexin subunits are illustrated in various configurations. Hemichannels may be homomeric (comprising six identical connexin subunits) or heteromeric (comprising more than one isoform of connexins). Hemichannels associate end to end to form the double membrane spanning gap junction channel. Channels may be homotypic (if connexons are identical) or heterotypic (if the two connexons are different) (adapted from Kumar and Gilula, 1996).

tions which support certain conclusions. We do not intend to review in-depth the current state of gap junction research, but rather focus on functional aspects of connexins in different organs and stages of development.

Mouse Connexin Genes

In the last decade, the targeted ablation of distinct connexin genes in the mouse genome yielded fundamental insights into *in vivo* function(s) of gap junctions. A prerequisite for the generation of these mouse mutants were extensive studies of mouse connexin genes. Different connexin genes were designated according to the species from which they were derived and the theoretical molecular mass (in kDa). For example, mCx26 means mouse connexin26, a protein of approximately 26 kDa (Beyer *et al.*, 1987). Mouse connexin proteins have been divided into several subgroups (α, β, γ, *etc*.) with respect to the degree of homology and length of their cytoplasmic loop (Söhl *et al.*, 2001b). The often used dendrograms of connexin genes, however, did not yield convincing evidence for the stepwise evolution of individual connexin genes, although this gene family is likely to have developed by gene duplication of ancestral connexin gene(s) (Bennett *et al.*, 1994). Most mouse connexin genes show a relatively simple structure, depicted in Figure 2A: an untranslated 5' exon1 is separated by an intron of variable size from exon2 harboring the complete reading frame and the 3' untranslated region. Until now, only three exceptions were found: first, for the connexin32 (Cx32) gene alternate promoter usage (Neuhaus *et al.*, 1995; Söhl *et al.*, 1996) in combination with at least three alternatively spliced 5' untranslated exon1 sequences (Söhl *et al.*, 2001a) was described (Figure 2B). Second, the reading frame of Cx36 has been shown to be located on exon1 and exon2, which are interrupted by an intron (Condorelli *et al.*, 1998; Söhl *et al.*, 1998) (Figure 2C); in this case, a hypothetical alternatively spliced exon1 might code for a heterogenous population of Cx36 proteins with different

N-termini. Third, analysis of Cx45 transcripts has revealed that the 5' UTR (untranslated region) is composed either of two exons1 and 2 or only of exon1 (the complete reading frame is located on exon3; Krüger *et al.*, 2000; Jacob *et al.*, 2001). This could imply functional consequences with respect to mRNA stability and/or translational control (Werner, 2000), similar to the alternative splicing of Cx32 (see Figure 2B and D). So far, splicing of connexin transcripts was only observed within the 5' region, not in the 3' region. In the Cx29 gene, the splice-acceptor and Kozak-consensus sequence for translational initiation nearly overlap, so that the Kozak-consensus sequence (Kozak, 1989) is restored by mRNA splicing (Söhl, unpublished), a feature that is unusual with other connexins, since splice-acceptor and Kozak-consensus sequences are normally separated by 10 to 20 bp on exon2. Moreover, clustered chromosomal location of certain mouse connexin genes has already been described several years ago (Schwarz *et al.*, 1992). Up to now, five connexin genes have been mapped, each on mouse chromosome 4 or on human chromosome 1, four of them are clustered within about 100 kb (*cf*. Table 1). Additionally, the Cx26, Cx30 and Cx46 genes, as well as Cx40 and Cx50 genes, are located close together on the same chromosome. Whether clustered connexin genes are commonly regulated remains to be determined. So far, no connexin pseudogenes have been detected in the mouse genome. The length of connexin mRNAs ranges from 1.6 to 8.5 kb (Table 1). Two hybridization signals of different size, as found with Cx30, Cx30.3 and Cx31 transcripts (Table 1), may imply different transcriptional initiation or termination sites as well as alternative splicing, which still need to be tested experimentally.

The topology of connexin proteins has been investigated only in case of Cx32, Cx26, and Cx43 proteins (Yeager and Nicholson, 1996; Yeager *et al.*, 1998), but is assumed

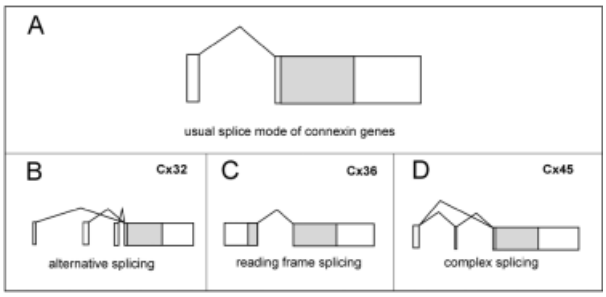

Fig. 2 Different Structures and Splice Patterns of Mouse Connexin Transcripts.
Shaded boxes represent the corresponding reading frames.
(A) Most of the connexin genes have a simple gene structure in which exon1 is separated by an intron from exon2, harboring the complete reading frame.
(B) Cx32 transcripts consist of at least three different exon1 sequences which could be independently spliced to exon2.
(C) In Cx36 transcripts the interrupted reading frame is restored by splicing.
(D) Splicing of Cx45 mRNA, in which the 5' UTR can consist of exon1 or a combination of exon1 and 2. The complete reading frame is located on exon3.

Table 1 Summary of Published Murine Connexin Genes.

Connexin	Subgroup	Intron [kb]	Chromosome	mRNA [kb]	Protein [aa]	Cyt. loop [aa]	C-term. [aa]	Reference
Cx26	β	3.8 (m)	14	2.4	226	35	18	1, 2, 4
Cx29		4.8 (m)	5	4.4	258	30	50	3
Cx30	β	n.d.	14	2.0, 2.3	261	35	55	1
Cx30.3	β	n.d.	4	1.8, 3.2	266	30	65	1
Cx31	β	2.3 (m) 2.6 (r)	4	1.9, 2.3	270	30	65	1, 5
Cx31.1	β	n.d.	4	1.6	271	30	70	1
Cx32	β	**B**: 4, 1.5, 0.3 (m)	X	1.6	283	35	75	6
Cx33	α	n.d.	X	2.3	286	55	60	1
Cx36		**C**: 1.2 (m, r)	2	2.9	321	100	50	7, 8
Cx37	α	1.0 (m)	4	1.7	333	55	105	1, 9
Cx40	α	11.4 (m) 5.5 (r)	3	3.5	358	55	135	1, 10, 11
Cx43	α	8.5 (m, r)	10	3.0	382	55	155	1, 12
Cx45	γ	**D**: 1.5, 1.5 (m)	11	2.2	396	80	150	1, 13
Cx46	α	n.d.	14	2.8	417	50	190	1
Cx47	γ	n.d.	11	2.5	437	105	155	14
Cx50	α	n.d.	3	8.5	441	50	210	1
Cx57	α	n.d.	4	3.5	505	55	275	15

Subgroup, intron length (if determined), chromosomal localization, mRNA size, protein length as well as the lengths of the corresponding cytoplasmic loop and C-terminal region are listed. m, mouse; r, rat; aa, amino acid residues. **B**, *cf*. Figure 2B; **C**, *cf*. Figure 2C; **D**, *cf*. Figure 2D. Cx29 and Cx36 do not fit in the α, β or γ-subgroup of connexins; n.d., not described.
References: (1) Rousset, 1996; (2) Kiang *et al.*, 1997; (3) Söhl *et al.*, 2001b; (4) Hennemann *et al.*, 1992a; (5) Gabriel *et al.*, 2001; (6) Söhl *et al.*, 2001a; (7) Söhl *et al.*, 1998; (8) Condorelli *et al.*, 1998; (9) Seul and Beyer, 2000; (10) Seul *et al.*, 1997; (11) Groenewegen *et al.*, 1998; (12) Yu *et al.*, 1994; (13) Jacob and Beyer, 2001; (14) Teubner *et al.*, 2001; (15) Manthey *et al.*, 1999.

to be similar for other connexins. The polypeptide chain of each connexin transverses the plasma membrane four times (Figure 1C). The cytoplasmic domains have highly divergent amino acid sequences, and the two extracellular loops are much more conserved among connexin proteins. The Cx57 protein, presumably the largest mouse connexin, should be twice as long as the Cx26 protein, the shortest one. The C-terminus of Cx57 is almost as long as the total Cx26, Cx29 or Cx30 polypeptides. In general, the C-terminal region has the largest variability in size (1:15) among all murine and human connexin domains, whereas the cytoplasmic loops could be divided into three categories: (i) small [30 – 35 amino acid residues (aa)], (ii) medium (50 – 55 aa), and (iii) large (80 aa, 100 aa or 105 aa; see Table 1). All other topological regions of the connexin proteins are more or less similar in size and show high degrees of sequence identity. The distances between the three characteristic cysteine residues within each extracellular loop are conserved among all connexin proteins: 1. loop (CX_6CX_3C) and 2. loop (CX_4CX_5C) except for Cx31 with (CX_5CX_5C) in the second loop (Hennemann *et al.*, 1992b). It was demonstrated that phosphorylation of different amino acid residues within the C-terminal region can modulate the gating probability of gap junctional channels or their half-life time (Lampe and Lau, 2000). After a screen of the Celera mouse genomic library (Eiberger *et al.*, 2001), we detected a novel connexin gene, termed mCx30.2, with highest homology to its putative human ortholog hCx31.9 (Nielsen *et al.*, 2000). A second novel mouse connexin gene, termed mCx39, was found to be orthologous to human Cx40.1, also a novel human connexin gene (Table 2). Interestingly, both genes (mCx39 and hCx40.1) seem to have an interrupted coding region, similar to the structure of Cx36 (Figure 2C). Whether both open reading frames are functionally expressed remains to be clarified.

Human Connexin Genes

Human connexin genes often show high sequence identity to their mouse orthologs. In addition, the molecular mass is often very similar, so that the same abbreviation can be used (*i.e*. hCx43 and mCx43). There are many exceptions to this rule, however: [mCx29 (258 aa) – hCx30.2 (270 aa)]; [mCx30.2 (278 aa) – hCx31.9 (294 aa)]; [mCx31.1 (271 aa) – hCx31.1 (270 aa)]; [mCx36 (321 aa) – hCx36 (322 aa)]; [mCx46 (417 aa) – hCx46 (435 aa)]; [mCx47 (437 aa) – hCx47 (436 aa)]; [mCx50 (441 aa) – hCx50 (432 aa)]; [mCx57 (505 aa) – hCx62 (543 aa)]. Interestingly, for mCx33 we did not detect a corresponding orthologous gene in the human genome, whereas we found two novel putative human connexin genes which do not have an obvious ortholog in the mouse genome: hCx25, and hCx59 (Table 2). The putative hCx40.1 gene appears to be the second human connexin gene (besides hCx36) that shows an interrupted coding region. In addition, we found two human connexin pseudogenes in the genomic data base. One of them, ψhCx43, had been identified several years ago (Fishman *et al.*, 1991) and the other one, ψhCx30.2, very recently (Söhl *et al.*, 2001b). Finally, the question remains: if it can be confirmed that the human genome contains additional connexin genes not present in the mouse genome, what is the functional impact of these genes and do they also occur in other species closer to man? Furthermore, the nomenclature of connexin genes should be improved. Many of the novel connexin genes in the mouse and human genome code for proteins around 30 kDa (*i.e*. hCx31.9 is orthologous to mCx30.2, and hCx30.2 is orthologous to mCx29; see Table 2). In addition, the mouse and human genes Cx30, Cx30.3, Cx31 and Cx31.1 have already been described. This situation should be simplified by introducing a new nomenclature.

Phenotypes of Targeted Connexin Defects in Transgenic Mice

Most cell types in mice or humans express more than one isoform of connexin. In most of these cases it is not clear to what extent these connexin proteins interact in primary cells to form heterotypic or heteromeric gap junction channels (Figure 1D). Since the ultimate goal is to understand the specific contribution of each connexin protein to the function of gap junctions in molecular physiology of a given tissue, we discuss here phenotypic alterations investigated in connexin deficient mice with regard to different cell types or organs, during embryonic development and in the adult organism.

Embryonic Development

The first targeted connexin defect described (Reaume *et al.*, 1995) led to altered cardiac morphology and perinatal death in Cx43-deficient mice. Obstruction of the right ventricular outflow tract of the heart prevented regular blood flow into the lungs and hence limited the blood oxygenation. There are some indications that alterations of cardiac neural crest cells are involved in the development of these heart malformations. Subsets of neural crest cells from the posterior rhombencephalon migrate to the outflow tract of the developing heart, where they become incorporated into the aorticopulmonary septum and the conotruncal cushions (Maschhoff and Baldwin, 2000). Apparently, neural crest cells migrate as cellular ensembles connected by Cx43 gap junction channels. Migration of cardiac neural crest cells was retarded in Cx43 deficient mice (Lo *et al.*, 1999). Overexpression of Cx43 using CMV promoter/enhancer elements, which direct expression predominantly in neural crest derivates, led to embryonic cranial neural tube defects and heart malformations (Lo, 1996). However, malformations in the Cx43-deficient and in Cx43 overexpressing mice are structurally different (Ya *et al.*, 1998). Targeted double connexin mutants defective in Cx40 and Cx43 (*i.e.* $Cx40^{+/-}/Cx43^{+/-}$ and $Cx40^{-/-}/Cx43^{+/-}$) exhibited a variety of heart malformations. Most charac-

Table 2 Summary of Currently Known Mouse and Human Connexin Genes (Listed in the Same Line) for Direct Comparison of Orthologous Genes.

Mouse connexin	Major expression	Phenotype(s) of Cx-deficient mice	Human hereditary disease(s)	Human connexin
	n. a.	n. a.	n. a.	hCx25
mCx26	breast, skin, cochlea, liver, placenta	lethal on ED 11	sensorineural hearing loss, palmoplantar hyperkeratosis	hCx26
mCx29	myelinated cells	n. a.	n. a.	hCx30.2
mCx30	skin, brain, cochlea	hearing impairment	nonsyndromic hearing loss, hydrotic ectodermal dysplasia	hCx30
mCx30.2	n. a.	n. a.	n. a.	hCx31.9
mCx30.3	skin	n. a.	erythrokeratodermia variabilis (EKV)	hCx30.3
mCx31	skin, placenta	transient placental dysmorphogenesis	hearing impairment, erythrokeratodermia variabilis (EKV)	hCx31
mCx31.1	skin	n. a.	n. a.	hCx31.1
mCx32	liver, Schwann cells, oligodendrocytes	decreased glycogen degradation, increased liver carcinogenesis	CMTX, (hereditary peripheral neurophathy)	hCx32
mCx33	testis	n. a.		
mCx36	neurons	visual deficits	n. a.	hCx36
mCx37	endothelium	female sterility	n. a.	hCx37
mCx39	n. a.	n. a.	n. a.	hCx40.1
mCx40	heart, endothelium	atrial arrhythmias	n. a.	hCx40
mCx43	many cell types	heart malformation	visceroatrial heterotaxia	hCx43
mCx45	heart, smooth muscle, neurons	lethal on ED 10.5	n. a.	hCx45
mCx46	lens	zonular nuclear cataract	congenital cataract	hCx46
mCx47	brain, spinal cord	n. a.	n. a.	hCx47
mCx50	lens	microphthalmia, zonular pulverulent and congenital cataract	zonular pulverulent cataract	hCx50
	n. a.	n. a.	n. a.	hCx59
mCx57	ovaries	n. a.	n. a.	hCx62

Note that for mCx30.2 (acc. no. AJ414561), mCx39 (acc. no. AJ414562), hCx25 (acc. no. AJ414563), hCx30.2 (Söhl *et al.*, 2001b), hCx40.1 (acc. no. AJ414564), hCx59 (acc. no. AF179597), and hCx62 (acc. no. AJ414565) only open reading frames have been found in the data bases and the actual patterns of expression remain to be experimentally determined. The accession numbers (acc. no.) refer to the genomic data base GenBank/EMBL/DDBJ and NCI. The bio-computive techniques used to identify the new connexin sequences and more information about homologous and orthologous relationships as well as the criteria adopted to classify the mouse and human sequences within the connexin family have been described by Eiberger *et al.* (2001). n.a., not analyzed; ED, embryonic day. Very recently, the human cDNA sequence of hCx32.4 has been deposited in the data base (acc. no. XM_064450). It is identical in its 5'part to the genomic sequence of hCx31.9 but differs in the remaining 3'part. No genomic sequence of hCx32.4 has been detected in the human genomic data base. It remains to be shown whether hCx32.4 represents a new human gene, a splice isoform or a cloning artifact.

teristic were defects at the atrioventricular (AV) junction. Growth of the right ventricle, rightward expansion of the AV junction, and septation were inhibited to various degrees (Kirchhoff *et al.*, 2000). In addition, slowed atrial depolarization as well as ventricular de- and repolarization were observed. These effects were additive in the ventricles, but not in the atria, probably due to overlapping expression patterns of Cx40 and Cx43 in the distal part of the ventricular conductive myocardium.

When the Cx45 coding sequence was deleted, the resulting mouse embryos died around ED (embryonic day) 10.5 showing an altered cardiac cushion (Kumai *et al.*, 2000) and altered blood vessel formation in the yolk sac, placenta, and embryo proper (Krüger *et al.*, 2000). The left ventricle of the heart was enlarged, probably due to high pressure as a consequence of narrower blood vessels (Krüger *et al.*, 2000). Expression of the Ca^{2+}/calcineurin dependent transcription factor NF-ATc1 (Kumai *et al.*, 2000) and growth factor TGFβ1 (Krüger *et al.*, 2000) was diminished in Cx45-deficient mice. It is unclear yet

whether both phenotypic alterations observed in the Cx45 deficient mice occur independently of each other, and how this led to death of the embryos.

Double mutants, defective in Cx43 and Cx32, did not die earlier than Cx43$^{-/-}$ mice. Tissues in which both genes were expressed in the same cell type developed normally until death of the embryos (Houghton *et al.*, 1999).

Targeted ablation of Cx26 coding DNA also led to embryonic death. In the labyrinth region of the mouse placenta, two adjacent cell layers (syncytiotrophoblast I and II) separate embryonic from maternal blood vessels. The two cell layers are connected by Cx26 containing gap junction channels which appear to function by allowing diffusion of glucose phosphate and other metabolites derived from the nutrients in maternal blood to embryonic blood and/or removal of waste products from embryonic blood (Gabriel *et al.*, 1998).

Recently, it has been described that Cx31 deficient mice (Cx31$^{-/-}$) were born at less than half of the expected Mendelian frequency (Plum *et al.*, 2001). Our detailed histochemical analysis of these mouse embryos revealed that the development of spongiotrophoblast cells was strongly retarded in all Cx31$^{-/-}$ embryos around ED 10.5. At ED 12, Cx43 expression was upregulated in the same cell type in which Cx31 was missing. Apparently, Cx43 can rescue the Cx31 defect in more than half of the Cx31$^{-/-}$ embryos (Plum *et al.*, 2001).

Since many of the mouse connexins have been found to be expressed in early embryogenesis, it is surprising that only relatively few connexin defects lead to a halt of embryonic development. It is assumed that, due to this redundant expression, different connexin proteins may compensate for each other during development, thus overcoming the defect of targeted deletion of single or double connexin genes (for a recent review, see Kidder and Winterhager, 2001).

Development of Germ Cells

Deletion of the Cx37 coding region led to a defect in oocyte development before meiotic competence was reached (Simon *et al.*, 1997). No transfer of Lucifer Yellow, when microinjected into Cx37$^{-/-}$ oocytes, to granulosa cells of the surrounding follicle cells was observed. This is in line with the notion that an unknown inhibitory signal (presumably cAMP) may be transferred from wild-type oocytes through Cx37-containing gap junctions to granulosa cells, thus preventing corpus luteum formation. The connexin isoform in hemichannels of the granulosa cell membrane which can dock to Cx37 hemichannels in the oocyte membrane is not known.

Cx43 expression is necessary for proper development of the ovaries. This has been shown by detailed histochemical analysis of transplanted Cx43$^{-/-}$ embryos (Juneja *et al.*, 1999). It is not known which step in ovarian development is defective, and how this is caused by the absence of Cx43. When the Cx43 coding region was replaced by Cx40 or Cx32 coding DNA, *i.e.* in Cx43 knock-in Cx40 (Cx43KI40) or Cx43 knock-in Cx32 (Cx43KI32) mice (Plum *et al.*, 2000), the resulting animals were viable but infertile. The corresponding males lack spermatocytes, but the reason for the nearly total infertility of female mice is not yet known. No morphological abnormalities in the generation of female germ cells were found (Plum *et al.*, 2000). Perhaps synchronized production and secretion of sexual hormones is impaired by lack of proper gap junctional coupling.

Heart and Blood Vessels

Homozygous Cx40 deficiency led to delayed impulse propagation in the conductive myocardium, as shown by an extended P wave and QRS complex in the electrocardiogram (Kirchhoff *et al.*, 1998; Simon *et al.*, 1998). Cx40 and Cx45 are both expressed in cardiomyocytes of the atrium and conductive system, whereas Cx43 is mainly expressed in the working myocardium. By high resolution optical mapping (Tamaddon *et al.*, 2000) or multielectrode array mapping (van Rijen *et al.*, 2001), a right bundle branch block has been observed in Cx40$^{-/-}$ mice.

Spontaneous atrium-related arrhythmias have been found in about 20% of the Cx40-deficient animals (Kirchhoff *et al.*, 1998; Hagendorff *et al.*, 1999) but not in Cx40$^{-/-}$ mice independently generated and analyzed by Simon *et al.* (1998), even after studying long-term electrocardiogram (ECG) recordings (Bevilacqua *et al.*, 2000). Most likely, these different findings were due to differences in the genetic background of the transgenic Cx40$^{-/-}$ mice (C57BL/6 and 129 Sv). This points to the influence of 'modifier' genes, which may impair heart function in Cx40$^{-/-}$ mice. Furthermore, conduction abnormalities, induced atrial arrhythmias and increased atrial vulnerability have been analyzed in Cx40$^{-/-}$ mice (Hagendorff *et al.*, 1999; Verheule *et al.*, 1999).

De Wit *et al.* (2000) have studied the propagation of vasodilatory signals along arterioles of the cremaster muscle in male Cx40$^{-/-}$ mice, triggered by local application of acetylcholine. They found that the amplitude of these signals was much more diminished in Cx40$^{-/-}$ animals than in wild-type animals. It is not clear whether these effects are due to defective intercellular communication between endothelial cells or between vascular smooth muscle cells. Theis *et al.* (2001) have generated 'floxed' Cx43 mice, in which the coding region of Cx43 was flanked by loxP recognition sites for Cre-mediated recombination. When these mice were crossed with TIE2-Cre mice that express Cre under control of gene regulatory elements of mouse endothelial cell surface receptor tyrosine kinase in the endothelium, we observed no effect on blood pressure and impulse propagation along arterioles in cremaster muscle. The deletion of the Cx43 gene was endothelium-specific and reached about 100%, as documented by expression of a lacZ reporter gene that was activated when the Cx43 coding region was deleted (Theis *et al.*, 2001). Surprisingly, Liao *et al.* (2001) have very recently described that independently generated floxed Cx43

mice (without *lacZ* reporter gene) after crossing with TIE-2-Cre mice led to progeny which was hypotonic and showed lower heart activity. One reason for these discrepancies could be the use of different TIE2-Cre mouse lines.

Recently, we have found that the intercellular transfer of Lucifer Yellow microinjected into endothelial cells of the opened visceral aorta was absent in the $Cx40^{-/-}$ tissue but occured in wild-type aortic endothelium. Transfer of microinjected neurobiotin was observed in both wild-type and Cx40-deficient aortic endothelium (Krüger *et al.*, 2002). Although these tracer molecules are not physiological, our results suggest that there may be some unknown specificity in diffusion of small metabolites through gap junctions formed by different connexins in primary tissue.

Vaidya *et al.*, 2001, reported reduced ventricular conduction velocity in the ventricles of $Cx43^{-/-}$ embryos starting at ED 15.5. Examined neonatal hearts exhibited arrhythmias *in vivo* as well as *ex vivo*. Gutstein *et al.* (2001) have also generated floxed Cx43 mice and crossed them with α-myosin heavy chain promotor Cre mice to achieve cardiomyocyte-specific deletion of Cx43. The progeny mice lived up to three month after birth and probably died because of sudden arrhythmias. Optical mapping analyses of the impulse propagation in these heart revealed slowed ventricular conduction in transverse and longitudinal direction. Since no morphological abnormalities could be found in the conditional knock out hearts, the authors concluded that there is no intrinsic cardiomyocyte cell-autonomous requirement for Cx43 during heart development.

We have carried out similar studies using the Cx43 floxed mice (Theis *et al.*, 2001) and the same α-myosin heavy chain promoter Cre mouse line used by Gutstein *et al.* (2001). Interestingly, mice that were homozygously deficient for Cx43 in cardiomycytes died within two weeks after birth and showed a variety of heart malformations including cardiomegaly. Surface ECG recordings revealed a severe decrease of the QRS-amplitude (Eckardt *et al.*, unpublished). Differences in the genetic background or the targeting constructs used for the generation of the floxed Cx43 mouse lines may account for the phenotypic differences described by Gutstein *et al.* (2001) and Eckardt *et al.* (unpublished). At present it is not certain whether there is a cardiomyocyte intrinsic requirement for Cx43 expression during heart development.

Although analyses of connexin function in the heart are greatly facilitated by measuring electrocardiograms in living mice and mapping of potential differences in isolated hearts, we are only at the beginning to understand the detailed contribution of different connexins expressed in the heart to the function of this organ. In myocardium, it is particularly important to distinguish between effects resulting from malformation during development and effects caused by functionally defective cardiomyocytes in the adult mouse. For this purpose, eventually all connexins expressed in the heart need to be conditionally ablated using cell-type specific and inducible gene deletions.

Liver and Pancreas

In mouse hepatocytes, Cx32, Cx26 and possibly Cx43 protein are expressed at low abundance next to bile canaliculi. Cx32 deficient mice show 78% decreased release of glucose from glycogen upon electrical stimulation of sympathic nerves (Nelles *et al.*, 1996). Electrostimulation caused a release of noradrenaline at the endings of sympathetic nerves. Noradrenaline triggers the release of inositol 1,4,5-trisphosphate (IP_3) inside of hepatocytes, which stimulates the mobilization of glucose from glycogen. IP_3 can pass through Cx32 channels about four-times more efficiently than through Cx26 channels in transfected HeLa cells (Niessen *et al.*, 2000a). Furthermore, cultured wild-type hepatocytes show 20-fold more intercellular transfer of IP_3 than Cx32 deficient hepatocytes (Niessen *et al.*, 2000b).

In primary hepatocytes, cultured from $Cx32^{-/-}$ embryos, total electrical conductance between cells is decreased by more than 80% compared to wild-type mouse embryos (Valiunas *et al.*, 1999). The regular course of liver regeneration after two thirds partial hepatectomy does not appear to be affected in Cx32 deficient liver but the synchronization of DNA synthesis during the initiation and terminating phase was less efficient than in wild-type liver (Temme *et al.*, 2000).

Recently, we have demonstrated that the secretion of bile from canaliculi in the liver following electrical stimulation was less attenuated in Cx32-deficient than in wild-type liver (Temme *et al.*, 2001). This suggests that the coordinated expulsion of bile by contraction of hepatocytes around bile canaliculi was impaired in Cx32 deficient liver.

In a previous study using Cx32 deficient mice that express no Cx32 protein in exocrine pancreatic acinar cells, we found out that the secretion of β-amylase was increased about two-fold (Chanson *et al.*, 1998). This was not unexpected, since it had been previously shown that secretion of this protein from acinar cells was inversely related to the level of gap junctional communication (Meda, 1996).

In contrast, secretion of insulin from endocrine pancreatic β-cells is directly proportional to gap junctional communication among these cells (Charollais *et al.*, 2000). Very recently, Calabrese *et al.* (2001) have described preliminary results that glucose induced secretion of insulin was lower in Cx36 deficient mice than in wild-type mice. Neither negative nor positive signals affecting secretion in pancreas by gap junctional communication have been molecularly identified. Ectopic overexpression of Cx32 in pancreatic β-cells does affect secretion (Charollais *et al.*, 2000).

Inner Ear

Connexin26 and 30 are coexpressed in supporting cells and fibrocytes in the inner ear (Lautermann *et al.*, 1998). It

has been hypothesized that these gap junctional channels could play a role in intercellular removal of K^+ and glutamate secreted from activated hair cells and taken up by neighboring supporting cells. We have tested this hypothesis in two different experimental systems. First, we have ablated Cx26 by gene targeting specifically in epithelial supporting cells of the inner ear (and, thus, circumvented the lethal phenotype of general Cx26 deficient mice; Gabriel *et al.*, 1998) by breeding floxed Cx26 mice with mice in which the Cre recombinase is activated by regulatory sequences of the otogelin gene. These mice are deaf and showed progressive disorganization of the epithelium in the cochlea, possibly due to apoptosis (Cohen-Salmon *et al.*, unpublished).

Second, Cx30 deficient mice, generated in our laboratory, also showed severe hearing impairment but the physiological and histological phenotypic alterations were different from Cx26 deficient mice (Teubner *et al.*, unpublished). Interestingly, in adult Cx26 deficient mice, the endolymphatic potential (between endolymph and perilymph) was lower than in wild type mice, in accordance with morphological degeneration of sensory epithelium in the inner ear (Cohen-Salmon *et al.*, unpublished).

It is not yet clear why inner ear-specific Cx26 mutants and conventional Cx30-deficient mice both exhibit severe hearing impairment, although both proteins appear to be coexpressed in the same cell types (Lautermann *et al.*, 1998). Perhaps the two connexin channels cannot fully complement each other, and/or heteromeric Cx26/Cx30 channels (see Figure 1D) exhibit new properties different from those of Cx26 and Cx30 homomeric channels (Manthey *et al.*, 2001).

Eye Lens

Two connexins, Cx46 and Cx50, are expressed in lens fiber cells. In addition, Cx50 seems also expressed in lens epithelium. Cx46 deficient mice develop nuclear lens cataracts at three weeks of age. Cx46 deficiency is associated with the occurrence of a proteolytic cleavage product of γ-crystallin in lens fiber cells (Gong *et al.*, 1997). Apparently, intercellular diffusion of metabolites or ions (*i.e.* Ca^{2+}) regulates the activity of a recently characterized calcium-dependent cystein protease LP82 and maintains the supramolecular organization of lens proteins responsible for lens transparency (Gong *et al.*, 1997; Baruch *et al.*, 2001).

Cx50 deficiency in mice also leads to cataract formation in the lens that is already evident one week following birth. Furthermore, the eyes of $Cx50^{-/-}$ mice are smaller and weigh only 56% of those of wild-type littermates (White *et al.*, 1998). Thus, although both Cx46 and Cx50 serve to maintain lens transparency, Cx50 must also be involved in morphogenesis of the lens. The molecular signals that diffuse through these channels and are responsible for phenotypical alterations compared to wild-type mice are not yet known.

Brain and Retina

Recently, we have generated mice in which the floxed Cx43 coding DNA can be deleted by Cre recombinase under control of glial fibrillary acid protein (GFAP) gene regulatory sequences. The resulting mice show a significant 20% increase of spreading depression (SD), a pathophysiological phenomenon characterized by a wave of depolarization followed by neuronal inactivation. This suggests that astrocytic gap junctional intercellular communication contributes to SD that has previously been interpreted as neuronal defect. An accelerated SD might indicate a role for Cx43 in the clearance of potassium ions from the extracellular space and implies involvement of astrocytic Cx43 in spatial buffering of potassium ions (Theis *et al.*, unpublished).

In the retina, connexin 36 is expressed at higher levels than in other organs and relative to other connexins that were found in this tissue (Güldenagel *et al.*, 2000; Söhl *et al.*, 2000). Cx36 is expressed in retinal AII amacrine and hippocampal interneurons as well as in excitable pancreatic β-cells (Güldenagel *et al.*, 2000; Serre-Beinier *et al.*, 2000; Venance *et al.*, 2000; Feigenspan *et al.*, 2001). Cx36-deficient mice are viable and fertile but exhibit an altered electroretinogram (ERG) which lacks the b-wave (Güldenagel *et al.*, 2001). This implies a severe reduction of the functional activity within the interneuronal network of the retina due to a loss of Cx36-mediated gap junction coupling. Furthermore, vision evoked potentials (VEPs) in the visual cortex were different between $Cx36^{-/-}$ and wild-type mice. At the same time, two other groups have studied the function of electrical synapses (*i.e*. gap junctions in excitable cells) in the cortex and hippocampus of Cx36-deficient mice. Deans *et al.* (2001) have generated mice in which two reporter genes were inserted instead of the deleted Cx36 coding DNA. They found Cx36 to be mainly expressed in cortical interneurons. The synchrony of the low-threshhold spiking of interneurons was much weaker and more spatially restricted in Cx36 deficient mice compared to control mice. Hormuzdi *et al.* (2001) have found that the γ-frequency network oscillations were disturbed in hippocampal slices of Cx36 deficient mice.

Human Connexin Mutants

In addition to the generation and characterization of targeted connexin defects in the mouse, the phenotypic alterations in identified human connexin mutants are leading to further insights into the molecular physiology of gap junctions. Many different mutations have been identified in the human Cx32 gene which cause *Charcot-Marie-Tooth disease (X-type)* (CMTX), an inherited demyelination disorder of the peripheral nervous system characterized by progressive wasting of distal muscles in the limbs (Bergoffen *et al.*, 1993). The Cx32 protein is expressed in Schwann cells, which are wrapped around the

axons of peripheral nerves. Cx32 containing gap junction channels connect the different layers of Schwann cells ('reflexive gap junctions'), thus forming conduits for direct signaling between the adaxonal area and the cell nucleus in the outer layer. Surprisingly, CMTX patients in general do not exhibit abnormalities in other organs (liver, pancreas, brain), where Cx32 is expressed in addition to peripheral nerves. It remains to be shown whether the defects described in Cx32 deficient mice can be functionally compensated by other connexins co-expressed in the corresponding human organs and cell types.

Mutations in the human Cx26 gene cause the most frequent non-syndromic sensorineural hearing defect in humans (Kelsell *et al.*, 1997). The corresponding conditional connexin26 deficient mice also show severe hearing impairment, and may thus be used as an animal model (Cohen-Salmon *et al.*, unpublished) of the human inherited disease. Some human Cx26 patients exhibit, in addition to the hearing defect, alterations in the epidermis (Richard *et al.*, 1998), where Cx26 is expressed in keratinocytes.

Two other human connexin defects in the Cx31 (Liu *et al.*, 2000) and Cx30 gene (Grifa *et al.*, 1999) also lead to hearing defects. Surprisingly, Cx31 deficient mice hear normally but show transient placental dysmorphogenesis (Plum *et al.*, 2001), which appears to be partially overcome by Cx43 expressed in the same cells. In contrast, Cx30 deficient mice show severe hearing impairment (Teubner *et al.*, unpublished) and, thus, may be useful as an animal model for the corresponding human inherited disease with regard to possible pharmaceutical intervention and future gene therapeutical approaches.

Defects in connexin26, 31, 30 or 30.3 (Macari *et al.*, 2000) have been described to cause the inherited human skin disease *Erythrokeratodermia variabilis* (EKV) (for a recent review see Richard, 2000). The molecular mechanism(s) of this genetically complex disorder have not yet been identified. Most of the corresponding connexin mutations concern single amino acid residues, which have dominant or transdominant effects (*cf*. Macari *et al.*, 2000; Rabionet *et al.*, 2000). Interestingly, a missense mutation in the Cx26 gene independently causes two different phenotypes in three unrelated families: mutilating keratoderma in combination with sensorineural deafness (*Vohwinkel's syndrome*) (Maestrini *et al.*, 1999).

Two different forms of cataracts in the human eye lens have been shown to be due to mutations in connexin genes. A congenital form of cataract is caused by mutations in the human Cx46 gene (Mackay *et al.*, 1999; Pal *et al.*, 1999), and zonular pulverulent cataracts have been found in patients who have a mutation in the Cx50 gene (Pal *et al.*, 1999). For each of these genes, deletion of the corresponding murine orthologous genes (mCx46 and mCx50), caused the corresponding types of cataracts, respectively (Gong *et al.*, 1997; White *et al.*, 1998). Thus, again these mouse mutants appear to closely mimic the corresponding human phenotypes.

Britz-Cunningham *et al.* (1995) reported single nucleotide exchanges in PCR-amplified Cx43 coding DNA from the original hearts of young children, who suffered from the visceroatrial heterotaxia syndrome and had undergone heart transplantation. Transfection of the mutated Cx43 coding DNA into a mouse cell line defective in gap junctional communication yielded differences in dye transfer compared to wild-type Cx43. The authors suggested that impaired junctional permeabilities of Cx43 channels in the original hearts of these patients could cause the symptoms of this disease. However, no Cx43 mutations were found in other cases of visceroatrial heterotaxia (Gebbia *et al.*, 1996; Penman-Splitt *et al.*, 1997). Thus, it is currently not clear whether visceroatrial heterotaxia can indeed be due to a defect in the Cx43 gene.

Outlook

Since the human and mouse genomes were sequenced to more than 95%, it is possible that all connexin sequences have been uncovered. This should form a basis for a new, rational nomenclature of connexins on which all gap junction researchers need to agree. Since we did not find any criteria to assign all connexin genes unequivocally to different subgroups of this gene family, we suggest that consecutive numbering of connexin proteins according to increasing molecular mass may provide the least biased nomenclature. Alternatively, one could group most of the connexins into three or four subgroups according to the extent of sequence identities and the length of the cytoplasmic loop (*cf*. Table 1).

From the characterization of targeted mouse connexin mutants and human patients suffering from a connexin defect, one can hypothesize that connexin proteins are likely to be involved in the following general functions: coordinated responses in cellular physiology (for example migration, contraction and secretion from cell ensembles), synchronized action of certain central neurons, intercellular balancing of second messenger concentrations above and below a threshold concentration triggering a response, removal of secreted ions and metabolites, and intercellular transit of nutrients or waste products after uptake into surrounding cells. In none of the organs and cells types which are defective in mouse or human connexin mutants has it been unequivocally clarified which ions and metabolites that can penetrate through gap junction channels are responsible for the effects observed. This problem remains one of the most difficult challenges in gap junction research.

Probably, each one of the connexin genes in the mouse genome will soon be experimentally deleted. It is unlikely, however, that this will clarify all questions regarding connexin functions. Since most cell types express more than one connexin, one can expect that there is functional redundancy of different connexin proteins. With regard to Cx43, Cx32, and Cx40, functional redundancy in cardiac morphogenesis as well as unique functions have been experimentally investigated (Plum *et al.*,

2000). We expect that much of the future research in connexin molecular biology will focus on clarifying how two or more different connexins can functionally interact and complement each other. In this context one has to find out how mouse connexin null mutants (that do not express the corresponding connexin protein) phenotypically differ from single base exchange mutants (which may code for dysfunctional connexin proteins). Possibly, the answer to this question will also provide an explanation for why the phenotypes of certain human connexin mutants (for example in Cx32) are largely different from the phenotypes obtained by deletion of the whole coding region as in most gene targeted connexin deficient mice.

At least two connexins (Cx36 and Cx39) appear to differ from the rest of this gene family by an interrupted coding region. The functional consequences of this exceptional gene structure are not yet clear. Finally, one needs to find out whether heteromeric or heterotypic connexin channels (Figure 1D) have different functional properties in certain cell types than homotypic gap junctional channels. It seems that understanding the interaction(s) between different connexins, as well as between connexins and unrelated binding proteins (such as ZO1, see Giepmans and Moolenaar, 1998; Toyofuku *et al.*, 1998) will lead to a new level of complexity regarding the molecular biology of connexin channels. In which way are connexins specialized, *i.e*. functionally different from each other? Do differences in connexin protein modifications such as phosphorylation, acylation, *etc*. lead to further specialization?

Regarding connexin expression in the brain (*i.e*. in neurons and glia cells) one can expect rapid progress in the near future. We need to understand how chemical and electrical synapses interact or influence each other topologically and functionally. Mammalian behaviour and memory may depend on the function of neuronal and glial gap junctions in the brain and in the peripheral nervous system. Connexins may fulfill a functional role in signal transduction through docking of hemichannels, which could be independent of the gating properties of the corresponding channels. Furthermore, connexin hemichannels may have an independent role, for example in extracellular release of ATP, that could bind to nucleotide receptors on neighboring cells. These and other speculations can be tested by appropriately engineered transgenic mice, but eventually all this information should serve to understand the function of connexins in our own body under healthy and pathological conditions.

Acknowledgements

We thank Gerda Hertig and Gabriele Matern for technical assistance. Work in the Bonn laboratory is supported by grants of the Deutsche Forschungsgemeinschaft, the Deutsche Krebshilfe, the Fritz-Thyssen Stiftung and the BIOMED Program of the EU. Novel connexin genes were identified in part by use of the Celera Discovery System and Celera Genomics' associated databases. Additional information is available under http://www.celera.com/publicationlibrary. We thank Elsevier Publishing for permission to use illustrations from the indicated references for preparation of Figure 1A–D. Furthermore, we thank Dr. Daniele Condorelli for help with the identification of hCx32.4

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