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Review

Defective gap junctional intercellular communication in the carcinogenic process

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Abstract

Gap junctions are membrane structures made of intercellular channels which permit the diffusion from cytoplasm to cytoplasm of small hydrophilic molecules. Nearly 40 years ago, the loss of functional gap junctions has been described in cancer cells and led to the hypothesis that such type of intercellular communication is involved in the carcinogenesis process. From this time, a lot of data has been accumulated confirming that gap junctions are frequently decreased or absent in cancer cells whatever their tissue and species origins. Here, we review such data by insisting on the possible links existing between altered gap-junctional intercellular communication capacity (or the altered expression of their constitutive proteins, the connexins) and the stages of cancer progression in various cancer models. Then, we analyse particular aspects of the disturbance of connexin-mediated communication in cancer such as the cytoplasmic localization of connexins, the lack of heterologous communication between cancer cells and normal cells, the role of connexin gene mutations in cancer. In a separate part of the review, we also analyse the disturbance of gap-junctional intercellular communication during the late stages of cancer (invasion and metastasis processes).

Keywords: Cancer; Carcinogenesis; Connexin; Gap junction; Gap-junctional intercellular communication

Contents

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1. Introduction

Gap junctions are present in all cell types of Vertebrates, except very few cases such as red blood cells, platelets, some neurons, mature skeletal muscle fibers and spermatozoids [1]. According to their ubiquity, it seems then reasonable, as it is generally admitted, to consider that gap junctions are fundamental structures necessary for cell differentiation [2,3], tissular physiology, and normal functions of the organs of the body [1].

Verifying, at least, this very general statement, physiologically abnormal cells such as cancer cells, can be added to the limited list of gap-junction deficient cells. Going through the literature in this domain, it is first surprising to see that gap-junction deficiency has been observed in cancer cells from a large panel of tissue and species origin; an observation supporting the general assumption that such a deficiency is associated to the cancer phenotype [4–6]. However, by considering precisely the literature, this general assumption may look more complex and less homogenous than expected depending (1) how gap-junction deficiency was defined and (2) on which cell or tissue models the observations have been carried out.

First, gap-junction deficiency has been defined in the literature from either the lack of gap-junction plaques through ultrastructural approaches (electron microscopy, freeze fracture) to the lack of gap-junctional intercellular communication (GJIC). GJIC itself has been defined with different meanings depending on the technical approaches used for estimating its capacity: electrical coupling, metabolic cooperation or dye-transfer assay. In this last case, the size and the biophysical properties of the tracers define the communication capacity even if they do not have any metabolic role. We may then argue about the concluding statement (lack of GJIC) when dye-transfer assay has been used but not electrical coupling. We may also argue about such a statement when the GJIC capacity of cancer cells and normal cells from the same origin has not been compared.

Secondly, it is legitimate to wonder whether it is possible to compare the GJIC estimations obtained from very different models such as in situ and ex vivo approaches or in vitro by using primary cells and established cell lines. Similarly, is the term “cancer” appropriate whatever we consider a sporadic tumor in human, a chemically-induced tumor in animals, primary cultures deriving from such tumors or established cell lines? Moreover, in addition to these different ways of estimating the GJIC capacity applied on these different (cancer) models, we have also to consider the animal species from which the tumor cells do originate. This last aspect is very important if we keep in mind that a rodent cell is much easier to transform and to become tumorigenic than a human cell. In other words, do we really get similar conclusions by using human or animal cells? However, despite these fundamental and important questions, the large amount of in vitro and in vivo data which has been accumulated do show that GJIC is frequently altered in cancer cells whatever their tissue and species origins.

The story nearly started 40 years ago. It was in 1966 that one of the first links between gap junctions and cancer was established when Loewenstein and Kanno reported a lack of electrical coupling in rat hepatomas. This phenomenon was observed in both chemically-induced hepatomas as well as in Morris and Novikoff’s rat transplanted hepatomas [7,8]; a situation which was completely different from the well-coupled normal liver cells [9]. Then, similar results were observed in

Table 1
Studies of the expression and/or function of connexins in various human tumor samples (tissue, primary cultures, cell lines)

Organ/tissue	Pathology	Study	GJIC	Cx expression	Reference
Bladder	Normal urothelial cells	Cell lines	+ (Scrape loading)	Cx26 and Cx43 (Northern);	[19]
				Cx26 ↑ (mRNA) in confluent cultures	[19]
	Cancer	Cell lines	–	Cx26 ↓ (mRNA)	[20]
	Normal urothelium	Tissue	NT[a]	Cx26[b] (punctuate staining in basal layer)	[20]
		Tissue	NT	Cx26 ↓ (70% of tumors)	
	Low grade non-invasive	Tissue	NT	Diffusely expressed (28%)	
		Tissue	NT	Heterogeneous loss of expression (44%)	
		Tissue	NT	Extensive loss of expression (28%)	
	High grade (invasive)	Tissue	NT	Diffuse expression (32%)	[19]
		Tissue	NT	Heterogeneous (41%)	
		Tissue	NT	Extensive (27%)	
Brain	Epilepsy	Primary culture	High (FRAP)[c]	Cx43 (intense)	[21]
	Low-grade astrocytomas	Primary culture	Moderate (FRAP)	Cx43 ↓ (moderate intensity)	[21]
	Glioblastoma multiforme	Primary culture	Lowest (FRAP)	Cx43 ↓ (low levels)	[21]
	Glioma (Grades I and II)	Tissue	NT	Cx43 (intense)	[22,23]
	Glioma (Grade III)	Tissue	NT	Cx43 (very weak)	
	Glioma (Grade IV)	Tissue	NT	Cx43 (almost not detectable)	[22,23]
Breast	Normal	Tissue	NT	Cx26 (−)	[24]
				Cx43 (punctuate staining in myoepithelial cells)	[24]
	Benign lesions	Tissue	NT	Cx43 (punctuate staining in myoepithelial cells)	[24]
	Ductal carcinoma	Tissue	NT	Cx43 (punctuate staining in myoepithelial cells)	[24]
	Lobular carcinoma	Tissue	NT	Cx26 (−), Cx43 (−)	[24]
	Mucoid carcinoma	Tissue	NT	Cx43 (punctuate)	[24]
	Invasive carcinomas	Tissue	NT	↑ Cx26 (cytoplasmic staining + heterogeneous: 15/27 samples);	[24]
				Cx43 (in stromal cells: 27/27 samples; heterogeneous expression in carcinoma cells and intracellular: 14/27 samples)	[24]
	Normal	Tissue	NT	Cx43 (+)	[25]
	Infiltrated and non-infiltrated ductal carcinoma	Tissue	NT	Cx43 (−)	[25]
	Infiltrated and non-infiltrated carcinoma	Tissue	NT	Cx43 (−)	[25]
	Carcinoma	Cell lines	NT	Cx43 (−) (4/6 cell lines by Western and Northern)	[25]
Cervix	Normal	Tissue	NT	Cx43	[26]
	Dysplastic regions	Tissue	NT	Cx43 ↓	[26]
Endometrium	Normal	Tissue	NT	Cx26 and Cx32[d]	[27,28]
	Hyperplasia	Tissue	NT	Cx26 and Cx32 (Weak or negative; 73–80%; Diffuse expression in cytoplasm: 20–27%)	[27,28]
				Cx43 (weak)	[27,28]
	Cancer	Tissue	NT	Cx26 and Cx32 (Weak or negative: 76–79%; Diffuse: 15–18%; Normal: 6%)	[27,28]
				Cx43 (weak)	[27,28]

(continued on next page)

Table 1 (*continued*)

Organ/tissue	Pathology	Study	GJIC	Cx expression	Reference
Head and neck	Squamous cell carcinomas	Primary cells	NT	↓Cx31.1 (cDNA microarray)	[29]
Larynx	Normal	Tissue	NT	Cx26, Cx30, Cx43[e]	[30]
	Squamous cell carcinoma	Tissue	NT	Cx26, Cx32, Cx43 (Heterogeneous expression)	[30]
Liver	Normal	Tissue	+[f]	Cx32 (+), Cx26 (+), Cx43 (−)	[31]
	Hepatocellular carcinomas	Tissue	↓[f]	Cx26 ↓/Cx32=cytoplasmic[g]	[31,32]
				Cx43 ↑ (cytoplasmic)	[31,33]
Lung	Normal	Tissue	NT	Cx26 (−), Cx32 (−), Cx43 (+)	[32]
	Carcinoma	Cell line	↓[h]	Cx43 ↓[i]	[34]
	Small-cell carcinoma	Tissue	NT	Cx26 ↑, Cx32 (−), Cx43 ↓	[32]
	Non-small cell carcinomas[j]	Tissue	NT	Cx43 ↓ Cx32 (−) poorly differentiated	[35]
		Freshly explanted tumor cells	−[k]	NT	[35]
		Cell lines	−[k]	NT	[35]
Oesophagus	Normal	Tissue	NT	Cx26 Cx43 (NT)	[36]
				Cx26[l] and Cx43[l]	[37]
				Cx26 and Cx43	[32]
	Squamous-cell carcinoma	Tissue	NT	Cx26 ↓[m] and Cx43 ↓[m]	[32]
Ovary	Normal	Primary cultures[n]	Extensive[h]	Cx43 (Cx26, 32, 37, 40: not detected)	[38]
		Tissue	NT	Cx43 cytoplasmic and punctated Cx26 (−), Cx32 (−)	[38,39]
	Adenocarcinoma	Cell lines	None or little[h]	None	[39]
				Cx43 (Northern and western) (stained positively: 59%)	
	Ovarian endometrioid adenocarcinomas	Tissue	NT	↑ Cx43 mRNA[o]	[40]
	Serous cystadenocarcinomas	Tissue	NT	Cx43 (↓) Cx26 (−), Cx32 (−)	[38]
				(19% stained positively for Cx43)	[39]
Prostate	Normal	Tissue	NT	Cx26(−) Cx32(−) Cx43(+)	[32]
	Benign tumors	Tissue	NT	Cx26 (NT) Cx32 (NT) Cx43(+)	[41]
	Cancer	Tissue	NT	Cx26 (NT) Cx32 (NT) Cx43↓	[41]
	Normal	Tissue	NT	Cx32 in cell–cell contact areas	[42]
	Tumors	Tissue	NT	Cx32 Cx43 in cell–cell contact areas (differentiated tumors) cytoplasm and loss in advanced stages (undifferentiated tumors)	[42]
	Normal	Tissue	NT	Cx43: basal epithelial cells	[43,44]
				Cx32: luminal epithelial cells	[43,44]
	Benign prostatic hyperplasia	Tissue		Cx32 ↑ and Cx43 ↑ (Increase of incidence and intensity in epithelial cells)	[43,44]
	Cancer	Tissue	NT	Cx43 (−): 65%	[43,44]
				Cx32 (−): 38%	
				Cx43 (−) Cx32 (−): 28%	
	In poorly differentiated cancer			Cx43 (−): 90%	[43,44]
				Cx32 (−): 60%	
	Normal	Cell lines	+[h]	Cx32 Cx40 transcripts	[45]
	Malignant	Cell lines	[p]	Cx43 transcripts GJIC	[45]
	Non-tumorigenic	Cell lines	[p]	Cx43	[46]
	Malignant	Cell lines	[h]	Cx43	[46]
	Normal	Epithelial primary cells	+[h]	Cx43	[47]
	Tumor	Cell lines	−[h]	↓ Cx43 (impaired post-translational modification)	[47]
Skin	Normal	Tissue	NT	Cx43[q]	[48]
	Basal cell carcinoma Squamous cell carcinoma	Tissue	NT	Cx43 ↓[r]	[48]
	Basal cell carcinoma	Tissue	NT	Cx43 ↓ and Cx26 ↑[s]	[32]

Table 1 (*continued*)

Organ/tissue	Pathology	Study	GJIC	Cx expression	Reference
Testes	Testes infiltrated	Tissue	NT	Cx43 Cx26 (−)	[49]
	Testes infiltrated with carcinoma in situ	Tissue	NT	Cx43 (−)	[49]
	Testes infiltrated with seminoma	Tissue	NT	Cx26 (cytoplasmic)	[49]
Thyroid	Normal	Tissue	NT	Presence of GJ (Freeze-fracture)	[50]
	Oncocytic adenoma	Tissue	NT	No GJ	
	Oncocytic carcinoma	Tissue	NT	No GJ	
	Papillary carcinoma	Tissue	NT	Presence of GJ	

[a] NT: not tested.
[b] When there is no indication on the techniques used the expression of connexins was studied by immunocyto(histo)chemistry or immunofluorescence, and the connexins are expressed.
[c] FRAP: Fluorescence Recovery After Photobleaching.
[d] Expression fluctuating during the phases of the reproductive cycle. Weak expression during the proliferation phase.
[e] Cx26 and Cx30 detected in parabasal and intermediate layers of the laryngeal epithelium. Cx43 detected in parabasal, basal and lower layers.
[f] Lucifer yellow transfer assay performed on fresh surgically removed samples.
[g] Only deficiency in normal punctate Cx32 and Cx26 staining was observed with altered localization of these proteins in some tumors.
[h] Microinjection of Lucifer yellow.
[i] Compared to non-transformed lung epithelial cells.
[j] Adenocarcinomas and squamous cell carcinomas.
[k] Estimation of GJIC by electroporation.
[l] Cx26 is specifically detected in the basal and intermediate layers of the squamous epithelium of esophagus. Similar but weaker pattern was observed for Cx43. Cx32 was not detected.
[m] Cx26 and Cx43 were coexpressed and confined to small areas in the tumor, whereas most parts of the tumors did not show any specific labeling. No significant decrease was observed between the primary tumor and the lymph-node metastasis [30].
[n] 2–4 passages of surface epithelium.
[o] Associated with deregulation of β-catenin.
[p] Scrape loading and FRAP.
[q] Cx43 was detected by immunoelectron microscopy and classical immunofluorescence. The expression varied according to the skin layers (weak expression in basal layer, increased expression in spinous layer and negative in horny layer).
[r] Small number of small gap junctions and cytoplasmic localization of the Cx43 (immunofluorescence and immunoelectron microcopy).
[s] Immunoflurescence heterogeneity of the Cx26 staining which looks more pronounced at the periphery of the tumors.

transplanted rat and hamster thyroid tumors [10]. The last example of this period came from human carcinoma of the stomach in which no electrical coupling was detected [11]. Already, in this short period of time, the same phenotype (lack of electrical coupling) appeared to be a common characteristic of solid tumors differently induced (chemically, transplanted or spontaneous) and originating both from various Mammal species (human, rat, hamster) and unrelated tissues (liver, thyroid, stomach). This first panel of data, coming from Loewenstein's laboratory and colleagues, established tumors, or cells derived from tumors, as communication deficient contrary to their normal counterparts. Since the most obvious phenotypic aberration of tumor cells is a deregulated growth, all these observations are at the origin of the general assumption that gap junctions are involved in cell growth control. The hypothesis linking lack of GJIC and cancer has been consigned in a review by W. Loewenstein himself [4]. Then, such an hypothesis has been reinforced by giving a more active involvement in carcinogenesis to gap junctions once tumor-promoting agents were found to be inhibitors of this type of cell-to-cell communication [12–15].

Now, it is known that the gap-junction channel (about 15 Å diameter) is expected to permit the cell-to-cell transmission of a wide range of cellular molecules (inorganic ions, metabolites, high-energy phosphates, nucleotides, cyclic nucleotides, second messengers, etc.) [16,17]. A priori, it would not be surprising to consider that such an ubiquitous and ancient intercellular channel adapted, through the Vertebrate evolution, to a wide variety of cellular functions involving the intercellular transfer of molecules; one of these functions being the intercellular transmission of growth-regulating signals. Forty years after the original observations, the hypothesis associating lack or diminished gap junctions and cancer is still valid and developing with new emerging concepts like the possible involvement of stem cells and their GJIC capacity in carcinogenesis [18].

In order to understand better how do gap junctions are involved in carcinogenesis, several in vitro and in vivo analyses then attempted to describe an association between disturbed connexin expression and particular stages of cancer progression. A review of such studies performed on human cancer materials is presented in Table 1. The object of this article is to review first such data not only obtained from human but also animal materials by insisting on the possible links that can be established between altered GJIC and the stages of cancer progression. Then, some particular aspects linking GJIC and cancer will be analysed such as the cytoplasmic localization of the connexins, the lack of homologous and heterologous communication among cancer cells, mutation of the connexin

genes and the involvement of GJIC in invasion and metastasis processes.

2. Connexins and cancer progression

An important fact to consider is whether the decrease of GJIC and/or connexin expression do follow the cancer progression. If such a relationship does exist, it may mean that connexins are involved in the carcinogenesis process; the progressive loss of GJIC favorizing the tumor progression.

2.1. Connexin loss as an early event of cancer progression

If a decreased expression of connexins has been often claimed in carcinogenesis; it is difficult to indicate at which step of the multistage process it really does occur. It has been suggested that tumors may derive from the clonal expansion of an adult stem cell that either does not express connexins or is sufficiently differentiated to express them. The first situation would explain why the cells do not communicate from the very early stages of tumorigenesis. The second situation would illustrate why cancer cells do express connexins at the early stages of carcinogenesis; the level of expression and/or function of connexins being then decreased by the onset of oncogenic activations at later stages of tumor progression. This so-called stem-cell concept has been extensively reviewed elsewhere [18].

In some cases, the decreased expression of connexins indeed seems to be an early event, occurring in dysplastic cells of precancerous lesions; which is hypothesized to contribute to their neoplastic progression. This is the case for Cx43 which is highly reduced in the dysplastic regions of the human cervix compared to the normal tissues [26]. Hyperplasia of endometrium also exhibits such an abnormal expression for Cx26 and Cx32 [28]. Similarly, the lack of detection of gap junctions by freeze fracture in thyroid tumors whatever their stages (adenomas and carcinomas) argues for an early event favorizing the clonal expansion of abnormal cells towards cancer [50].

An interesting example illustrating that a disturbed expression of connexins might be a prerequisite for human cell expansion could be kidney. Indeed, hemodialysis patients with end-stage renal disease have an increased incidence of renal cell carcinoma compared to the general population. Hypermethylation of CpG islands of the Cx32 gene has been observed in both cancerous and non-cancerous regions of the kidney from such patients. Since hypermethylation of the Cx32 gene occurred only in cancers lesions from patients of the general population, the consequent lack of expression of Cx32 would be related, or even a prerequisite, to the early stage of renal carcinogenesis [51]. However, if the decreased expression of connexins or the lack of gap junctions at early stages (adenomas or even dysplastic region of precancerous lesions) has been observed in a wide range of tissues, it cannot be a "general law". As it is often the case, the situation is more complicated and depends on the tissue which is considered.

For instance, in the larynx, no obvious difference of connexin expression has been reported; Cx26 (in parabasal and intermediate layers), Cx30 and Cx43 (in basal, parabasal and lower layers) have a similar level of expression in normal tissue and in precancerous lesions [30]. On the contrary, an hyperexpression is even observed in some dysplastic lesions of the larynx [30]. The aberrant expression was observed in later stages (squamous cell carcinomas) and characterized by a heterogenous staining for these connexins (regions with intensive expression alternated with region of no expression). In prostate, the decrease of Cx43 is more obvious in the late stages but not in the benign stages [41]. This would mean that the decreased Cx43 expression is not involved in the initiation of prostate cancer [41]. This hypothesis is reinforced by the fact that there is a marked increase in incidence and intensity of Cx43 immunostaining in benign prostatic hyperplasia [43,44]. A similar observation was made about Cx43 in human gliomas: three different studies reported so far a diminished expression of Cx43 which correlates with the progression of the tumors [21–23].

2.2. Liver cancer as a model of connexin-related cancer progression

Liver cancer is an interesting model for studying the possible involvement of connexins in cancer. Indeed, well-established protocols of chemically-induced liver cancer in rodents have been known for long and provided a cancer-progression model exhibiting well-defined stages. Since liver was known to be a well-coupled tissue, expressing at least two major connexins (Cx26 and Cx32), it has been extensively used to reveal any putative correlation between connexin disturbance and fundamental steps of liver carcinogenesis. Moreover, since liver tissue is pretty homogenous and soft, it became possible to perform ex vivo dye-transfer assays [52]. Such a functional approach permitted to have a rather complete set of tools not only for studying the function (ex vivo microinjection of fluorescent tracers) but also the fluctuation of expression at the mRNA and protein levels (Northern and Western analysis) and the localization (immunohistochemistry) of connexins during each of the well-defined stages of chemically-induced rat hepatocarcinogenesis. Finally, it was possible to use the same tools for human liver tumors in order to see if any disturbance of GJIC could be a general phenomenon independently of the considered species.

2.2.1. Human liver cancer

First, if we consider human liver cancer, connexin expression might be thought to not be a good marker of cancer progression since the decreased GJIC capacity which is in adenomas as strong as in carcinomas is not accompanied by a decreased expression of the connexins [31,33]. However, it is different if we consider the localization of Cx32; in adenomas it is detected in parts of the plasma membrane in contact with neighboring cells contrary to hepatocellular carcinomas in which Cx32 is mostly localized in the cytoplasm [31]. In addition to this aberrant expression and/or localization of the original connexins, another disturbance concerns the newly

synthesized cytoplasmic and non-phosphorylated form of Cx43 in the invasive parts of human liver. The newly expression of Cx43 in hepatocellular carcinomas can be the sign of a "dedifferentiation process". On the other hand, it can be also due to the presence of liver stem cells (oval cells) which are known to express Cx43 [18,53]. However, a study evoked the presence of both Cx32 and Cx43 in the cytoplasm and in the plasma membrane of normal human liver [54]. Despite this fundamental difference with other studies showing no detection of Cx43 in normal human liver, both connexins were found to be markedly decreased by these authors in the hepatocellular carcinomas at a post-translational level [54].

2.2.2. Rat liver cancer

2.2.2.1. Early stages of rat liver cancer. The picture is clearer for rat liver carcinogenesis. An early and progressive decreased expression of connexins is clearly observed in chemically-induced liver tumors. In rats, Cx32 mRNA is decreased in hyperplastic nodules induced by *N*,*N*-diethylnitrosamine (DEN) or N-ethyl-N-hydroxyethylnitrosamine (EHEN) treatments. It is barely detectable in further stages such as hepatocellular carcinomas [55]. When the function of gap junctions was tested by microinjecting fluorescent tracers such as Lucifer yellow, it was clear that the loss of Cx32 mRNA is accompanied by the decrease of GJIC. In such an experimental model, the decrease of GJIC appears to be an early event which is already obvious 4 weeks only after the beginning of the chemical treatment before the apparition of the focal lesions. Interestingly, the decreased GJIC capacity appeared before the decrease of the number of Cx32 spots as detected by immunohistochemistry meaning that a conformational change of Cx32 connexons could have been induced via phosphorylation by the treatment [52]. Most enzyme-altered (glutathione S-transferase placental form positive: GST-P positive) focal lesions showed lower GJIC and lower Cx32 spots than surrounding hepatocytes leading probably to a lack of heterologous communication which could emphasize the clonal expansion of such lesions.

An interesting observation is that if Cx32 mRNA is decreased in cells of the primary tumors induced chemically, the immunocytochemical analysis revealed a decrease in gap junctions in some but not all preneoplastic focal lesions [56]. Others described similar facts: only a small part (17%) of the GST-P positive foci were found to have a marked reduction of Cx32 gap junctions in rats; this decrease being more important in hyperplastic nodules [57]. Not such a relation was found for Cx26 which seems to be differently regulated at least in the first step of liver carcinogenesis. It is more expressed in some of the GST-P positive foci (44%) and in a small part of the hyperplastic nodules (16%) [57]. Similarly, most preneoplastic-altered foci generated by DEN initiation and phenobarbital or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) promotion exhibited a decreased Cx32 or an increased Cx26 staining; Cx43 being not detected [58]. Interestingly, the hyperexpression of Cx26 might be related to cell proliferation since Cx26 is enhanced in hepatocytes before the onset of S-phase after partial hepatectomy in rats [59]. Therefore, the Cx32 decrease is an early event in induced rat liver carcinogenesis; this conclusion is reinforced by the fact that the quantity of Cx32 spots is decreased after partial hepatectomy, a promotion stage of rat liver carcinogenesis. This decrease is probably due to a lower amount of Cx32 mRNA [59].

This observation may tend to indicate that the focal lesions exhibiting a low level of gap junctions are the precursors of the primary tumors; these primary tumors keeping a low level of gap junctions. If this scenario is true, it means that in the rat liver, the loss of gap junctions is a prerequisite for the further development of primary tumors. Such a picture might be a correct one since Cx32-knock out mice do present a higher level of both spontaneous and chemically-induced liver tumors [60]. Similarly, the highest sensitivity of female rats than males to hexachlorobenzene is related to a lower amount of Cx32 mRNA (8 fold lower in females than in males) [61].

2.2.2.2. Late stages of rat liver cancer. A progressive decrease of Cx32 expression is often observed from early preneoplasia (enzyme-altered foci) to hyperplastic nodules and hepatocellular carcinoma (no Cx32 is even detected in pulmonary metastatic hepatocellular carcinomas). Since there is an inverse correlation with an increase BrdU index, the observed decrease appears linked to the cell proliferation and progression of hepatocarcinogenesis [62]. The fact that the number of Cx32 positive spots per mm^2 is significantly less in hepatocellular carcinoma than in surrounding non-carcinomatous cirrhotic tissues [63] may suggest that the Cx32 loss provides a cellular independence and a growth advantage to tumor cells [62].

The deficiency of normal punctuate Cx32 staining may not be related to a loss of Cx32 mRNA but rather to an altered localization of the protein in some tumors [58]. In this last case, Cx32 exhibited some altered electrophoretic mobility suggesting that post-translational modifications are responsible for the altered localization. In some tumors, Cx32 mRNA was detected without corresponding Cx32 immunoreactivity, indicating that some hepatomas downregulate Cx32 independently of mRNA abundance [58]. This tends to show that several pathways may lead to a decreased level of GJIC which seems to be the common, final and crucial event, tightly controlled by the level of Cx32 mRNA expression, the post-transcriptional expression of Cx32, the localization of this connexin and possibly its phosphorylation state. These different levels of alteration of the Cx32 might be related to the rat strains or the treatments.

However, if Cx32 expression and/or function are highly related to the progression of rat hepatocarcinogenesis, we do not know how Cx32 does control cell proliferation. Cx32-KO mice exhibit a higher rate of liver tumors than wild-type mice [60]. Apparently, in these Cx32-KO mice, the amount of Cx26 is also decreased in the hepatocytes [60] up to a level which is probably not sufficient for suppressing the tumor growth. Recent observations have shown that Cx32 expression is needed for the initiation of synchronous DNA synthesis in hepatocyte nuclei after partial hepatectomy [64]. Since cAMP-

signaling pathway have been shown to be involved in liver regeneration in partially-hepatectomized rats [65], this synchronous activation of quiescent hepatocytes could be achieved by equilibrating second messengers (cAMP or IP3) among cells. Interestingly, CREM-deficient mice exhibit a significant reduced cell proliferation during liver regeneration [66].

Despite the fact that Cx26 is also decreased in hepatocellular carcinomas [57], it seems reasonable to postulate that Cx32 and Cx26 are differentially regulated during the progression of rat liver carcinogenesis. First, the amount of Cx32 is apparently decreased earlier than Cx26. Second, the onset of tumorigenesis is related to both a decrease of Cx32 expression and/or cell communication function and an increase of Cx26 expression (possibly at the S-phase of the cell cycle). Since the Cx26 overexpression does not seem to counteract on the communication capacity, we may postulate that the two connexins have different roles concerning their involvement in the regulation of communication in liver; Cx32 loss is clearly related to GJIC loss, independently of Cx26 expression.

2.3. Skin cancer

In skin, most extensive studies on connexin expression have been performed on the mouse model. These studies were mostly focused on the behaviour of Cx26 and Cx43 which have been thought for long time to be the major connexins of the skin tissue. Now, we know that the expression pattern of the connexins in skin is very complicated and concern other types of connexins which have been identified in more recent years. For these reasons, the "picture" of connexin expression related to skin carcinogenesis is not complete and still need further studies both in animal models and in human tissues.

2.3.1. Studies on murine and human skin tumors

In mouse skin, the pattern of expression of Cx26 and Cx43 is clearly segregated: Cx43 is predominantly expressed in the less differentiated lower spinous layers of the normal skin whereas Cx26 is more present in the terminally differentiating upper spinous and granular layers [67]. The particular pattern of expression of Cx26 and Cx43 does not seem to be altered in the hyperplastic epidermis. It starts to be modified from the papillomas stages and follows two fundamental steps: (1) a loss of segregation in papillomas (both Cx26 and Cx43 are localized in the lower spinous layers); (2) a loss of detection in the squamous cell carcinomas [67]. The decrease of Cx26 and Cx43 expressions is apparently related to the progression stages of the tumors from papillomas to well-, moderately- and poorly-differentiated squamous cell carcinomas of the skin induced chemically [68]. If a loss of segregation of expression is commonly observed for Cx26 and Cx43 in papillomas [67,68], some found an overexpression at this stage [69]. However, in late papillomas, a local loss of Cx26 immunostaining can be observed [69]. Then, the decrease of both connexins seems to be the consequence of a post-translational phenomenon since mRNA is still present [69]. Very few is known about the other connexins which are also present in the skin: a strong inhibition of Cx31.1 during all stages has been reported [69] but more studies are needed to complete the "connexin scenario" during chemically-induced skin carcinogenesis in mice.

Moreover, we have to consider that the rare studies on connexin expression which have been performed on human biopsies did not report a so clear picture. Apparently, Cx43 is poorly present in squamous cell carcinomas [48]. This is also the case in the basal cell carcinomas contrary to Cx26 which was even found to be more expressed in the human basal cell carcinomas than in the normal epidermis [32,48].

2.3.2. Studies on in vitro models of skin cancer

Cell lines and primary cultures from different stages of mouse skin carcinogenesis have been used to estimate whether the GJIC capacity is related to the progressive stages of cancer progression. In general, it has been reported a good correlation between the decrease of GJIC capacity (often tested by Lucifer yellow microinjection) and the progression of skin cancer [70,71].

However, such in vitro approaches have to be considered carefully since the establishment of cell lines may denaturate the original properties of the tumor cells. This may explain why no significant difference of GJIC was found sometimes between cell lines from normal keratinocytes, papillomas or squamous carcinomas [72]. Actually, for this last report, a marked decrease (80–90%) in GJIC was found on progression from squamous to spindle carcinoma cells. E-cadherin seems to be involved in the regulation of GJIC in such cells by permitting or not, depending on their level of expression, the correct addressing of the connexins towards the cell membrane [72,73].

2.4. Bladder cancer

2.4.1. Studies on human material

As for the two previous kinds of cancer (liver and skin), two connexins have to be considered: Cx26 and Cx43. Contrary to these two previous examples, the correlation between the connexin behaviour and the progression stage of the bladder cancer is not so clear and looks confusing depending on the models used. By considering human cancer cells lines, a loss of Cx26 expression has been associated with the malignant phenotype contrary to Cx43 whose variable expression in cancer cell lines is not related to the GJIC capacity [19]. The expression of Cx26 is decreased but heterogeneously in situ without any clear difference between non-invasive and invasive cancers [20]. However, others have found that Cx43 expression and GJIC capacity of human uroepithelial cells are inhibited by the exposure to a tobacco-related nitrosamine [74]. Interestingly, the expression at the protein level of Cx43 is recovered within 24 h of removal of the carcinogen [74]. According to these results, Cx43 could play a major role at the very precancerous stages and Cx26 a more crucial role at the following steps of cancer progression but the data are too parcellar to make such a conclusion as a definitive one.

2.4.2. Studies on rat material

Bladder carcinoma cells from rats present contradictory results compared to the human model. In such cells, there is a

clear tendency that all cell lines with a greater communication capacity (due to higher levels of Cx26 or Cx43 mRNAs) were the most tumorigenic. These results were similar to those obtained from in situ studies. In rat bladder, Cx43 is barely detectable and Cx26 is not. However, in rat bladder carcinomas, mostly in N-ethyl-N-(4-hydroxybutyl)nitrosamine (EHBN)-induced carcinomas, abundant expression of the two types of connexins was observed. It was even concluded from these studies that increased GJIC capacity or increased connexin expression may give a growth advantage in rat bladder carcinogenesis [75].

2.5. Oesophageal cancer

If there are contradictory observations for a same kind of cancer, such as bladder cancer, depending on the considered species, there are also cases, such as oesophageal cancer, for which the same connexins (Cx26 and Cx43) expression and/or function do not exhibit any correlation with cancer progression. No drastic loss of connexin expression was found in squamous cell carcinomas of the human oesophagus [32]. Cx26 and Cx43 were still detected immunohistochemically but the only difference was on the heterogenous staining: some parts of the tumors exhibiting a normal staining and other parts without any staining. This heterogeneity was not modified according to the tumor phase since it was also found in metastasis. It has been suggested that such a heterogeneity of connexin expression could be the consequence of cancer stem cells present within the tumors with their partially-differentiated daughter cells [18].

However, this apparently lack of correlation between connexin expression and the progression stage of the tumor is related to what is observed in cell lines; in such cells the level of expression does not correlate perfectly with tumorigenicity [37]. Such a lack of correlation was also observed in rat cells; both non-tumorigenic and tumorigenic oesophageal cell lines exhibited high level of dye coupling and comparable levels of Cx43 expression [76].

2.6. Prostate cancer

The picture is clearer for human prostate cancer in which Cx32 and Cx43 expressions have been studied. The decrease of expression for both connexins is obvious in the carcinomas and even stronger in the poorly-differentiated tumors [44]. The study realized by Mehta was more precise since he reported, with his colleagues, a correct localization of the two connexins in well-differentiated tumors and a more cytoplasmic localization in the undifferentiated ones with an eventual loss of expression in advanced stages [42]. If this is true, it would mean that these connexins do not play a role at the beginning of the progression of prostate cancer. Interestingly, more doubt concerns the expression of the connexin in the normal tissue. Some only found Cx32 detectable [42] when others found a segregated expression of Cx32 (in luminal cells) and Cx43 (in basal cells) [44]. In the first case, Cx32 was detected in the tumors, meaning that its expression (like Cx43 for liver cancer) would be associated with the dedifferentiation of the tissue. More studies are necessary in order to have a better picture of the connexin expression pattern in human prostate cancer.

2.7. Breast tumors

In human breast, the expression and localization of Cx26 and Cx43 have been studied. Interestingly, Cx26 was not detected in the normal tissue but looked upregulated and cytoplasmic in invasive lesions of breast carcinomas [24]. The pattern of expression was different for Cx43 with a heterogeneous expression at intercellular regions of the carcinoma cells in some of the tumors studied. Laird et al. only studied Cx43 which was not detected in lobular and ductal carcinomas whatever the grade tested [25]. The result was so clear that the authors concluded that Cx43 would be an interesting marker for early oncogenesis of the breast. Here, we have to emphasize the discrepancy of results which can be obtained depending on in situ or in vitro observations. Indeed, Cx26 was claimed to be a putative breast-tumor suppressor gene by using a cell model of human breast cancer [77]. Such a result is in contradiction with in situ observations [24].

2.8. Lung cancer

Decreased expression of Cx43 has been observed in various human and mouse lung carcinoma cell lines which exhibit lower dye-transfer capacity than non-transformed lung epithelial cells [34,78–80]. Similarly, lack of communication, as tested by electroporation of Lucifer yellow [81], is a common feature of human lung carcinoma cell lines or cells freshly explanted from human lung tumors [35]. However, positive controls for this study were fibroblasts (exhibiting GJIC) and not epithelial cells which would be a more appropriate control.

Lack or decreased expression of connexins is not always observed for lung cancer. In mouse, a study which compared by competitive cDNA library screening the gene expression in chemically-induced lung carcinomas and normal lungs did not report any change of connexin gene expression among the 22 clones which were found to be differentially expressed [82]. This is in agreement with another study performed with urethane-treated A/J mice. Primary cells obtained from hyperplasias, adenomas and carcinomas of these mice exhibited extensive dye-transfer even at late-stage carcinomas. The loss of GJIC was obtained after several months in culture, meaning that in vitro the propagation of tumor cells can lead to gap-junction closure [83]. These results suggest that the molecular changes that lead to the formation of the tumor in vivo are not sufficient to interrupt gap junctions. An alternative explanation to the loss of GJIC during the in vitro propagation would be the selection for a few non-communicating cells that were present in the original population. If this is true, we cannot exclude the hypothesis that such cells might be initiated and non-communicating stem cells as proposed by Trosko [18].

However, the decreased amount of Cx43 which was observed in the $Cx43^{+/-}$ mice makes them more sensitive to lung cancer after urethane treatment [84]. This last result tends

to show that the decreased amount of Cx43 could be a prerequisite leading to a deregulated growth of lung cells.

2.9. Discussions about the models used

The in vitro and in vivo/in situ studies presented above show that certain types of connexins may be specifically altered in some cancers. For instance, the loss of Cx26 expression seems to be associated with the malignant phenotype of human bladder cancer cells [19]. The loss of Cx32-mediated GJIC (by loss of mRNA in rats or cytoplasmic localization in humans) is associated with hepatocarcinogenesis [31,55]. The loss of Cx43 could be a marker of human breast cancer [25]. These results, in which different connexins seem to be involved in cancer affecting different organs, tend to show up a specific link between the growth regulation of one type of tissue with one type of connexin.

However, we have to be careful about these general conclusions. All these studies which are presented above do not reveal a simple and general aberration of GJIC in cancer. The picture is more complicated and seems to depend on the type of cancer which is considered. Except the case of liver which presents a rather homogenous picture concerning the connexin disturbance, the involvement of connexins depends on the model used. For instance, some discrepancies can be observed between in situ/in vivo analysis and in vitro studies. We may then wonder whether in vitro models such as cell lines are good models or not for studying the involvement of connexins in carcinogenesis. In particular, it is important to consider if the loss of gap junctions which is observed in vitro is actually associated with a neoplastic process, rather than being artificially induced by extensive cell culture: as it is for primary cells cultured from urethane-induced lung tumors [83]. Even if these cells were isolated from late-stage carcinomas, they possess an extensive GJIC capacity immediately upon isolation. The following propagation of these tumor cells in culture could induce either additional alterations that can lead to gap-junction closure or preferential in vitro clonal expansion of non-communicating cells originally present [18,83].

Moreover, connexin expression may depend on the cell environment. This was the case for hepatoma cells which fail to express connexin mRNAs in culture and express Cx32 mRNA once transplanted in vivo. However, after transplantation, Cx32 interestingly keeps being down regulated at the post-transcriptional level (Cx32 immunostaining is observed in less than 5% of the neoplastic cells in vivo) [85]. A shift of connexin expression is also observed: these cells (9618A cells) express Cx43 mRNA in vitro but Cx32 mRNA in vivo. This is different with other cells (N1S1 cells) which express Cx43 mRNA whatever their environment [85]. A similar phenomenon has been observed for mouse skin carcinoma cell lines unable to express Cx26 in vitro. Those cells growing as tumors in nude mice start to express Cx26 protein [71].

In some cases, a good correlation between in vitro models and tissues can be found for different kinds of tumors such as human ovarian carcinomas or rat liver carcinomas. In the first case, a lack of Cx43 expression was observed both in carcinoma cell lines [38] and in surgery pieces [39]. In the second case, the lack of expression of Cx32 which is observed in hepatocellular carcinomas is well correlated with the lack of expression of this connexin in hepatoma cell lines [86]. A similar good correlation between in vitro and in vivo models exists for rat bladder cancer but it goes in the opposite way concerning GJIC and remains an exception among other types of cancer: the chemically-induced rat bladder carcinomas exhibit an abundant expression of Cx43 and Cx26 whereas those connexins are barely detectable in the normal bladder tissue. Similarly, most tumorigenic rat bladder carcinoma cell lines exhibit an extended GJIC capacity related to both Cx26 and Cx43 expression [75].

In other cases such as human breast cancer, the data concerning Cx26 seem contradictory depending on the in vitro and in situ models. Cx26 was found to be a tumor suppressor in human breast cancer cells whereas it is not always detectable in normal breast tissue and upregulated in invasive lesions of breast carcinomas [24]. We may then conclude as S. Jamieson: “upregulated Cx26 in carcinoma cells is not necessarily inconsistent with a tumor suppressor role for GJIC. However, the role of gap junctions in the formation and progression of solid human tumors is likely to be more complex than indicated from experimental systems” [24]. In vitro models such as cancer cell lines are artificial indeed, but they present the advantage to minimize the number of uncontrolled parameters and they can bring important answers concerning the possible involvement of connexins in cell growth control.

3. Aberrant gap junctional intercellular communication and cancer

Aberrant GJIC can be either found among cancer cells or between cancer and normal cells. The lack of GJIC among cancer cells seems to be the consequence of two major phenomenons: either a lack of expression or an aberrant localization of the connexin proteins. The lack of expression is often the consequence of a lack of transcription which may be due to hypermethylation of the connexin normally expressed. So far, there are very few examples suggesting that such a phenomenon does happen. The downregulation of Cx32 expression by hypermethylation of the CpG island of Cx32 gene has been observed in human renal cell carcinomas and in a human renal cell carcinoma cell line [51,87]. The treatment of Cx43-negative HeLa cells with 5-aza-2′-deoxycytidine resulted in expression of Cx43, suggesting a Cx43-gene silencing via DNA methylation [88]. However, this is not always the case [36] and we still do not know precisely how connexin transcription is down regulated in some cancer cells.

3.1. Cytoplasmic localization of connexins

Several studies have shown that the expression of connexins can occur in tumor cells but are abnormally localized and accumulate in the cytoplasm. Such observations have been made both in vitro and in vivo and did not depend apparently on the origin of the tumor.

3.1.1. Cytoplasmic localization of connexins in vivo

In skin, Cx43 was detected by gold particles in small gap junctions but scattered in the cytoplasm of human basal cell carcinomas and squamous cell carcinomas [52]. This last observation was not made in normal skin. Similarly, Cx26 was found to be cytoplasmic in human invasive carcinomas of the breast in more than 50% of the cases [24]. This was also true for Cx26 in some human bladder tumors [20] and in Sertoli cells of testis infiltrated with carcinomas in situ [49]. In this last case, the situation is even more complex since in Sertoli cells the Cx26 expression was induced cytoplasmically when Cx43 expression was decreased. This was associated to a less differentiated stage of Sertoli cells as demonstrated by the re-expression of cytokeratin 18 [49]. The altered expression of Cx26 and Cx43 in Sertoli cells in testes infiltrated with carcinomas in situ or seminoma suggests that a derangement in intercellular communication between Sertoli cells (and between Sertoli cells and germ cells) may play a role in the resulting spermatogenic impairment and in the proliferation and progression of carcinomas in situ [49]. Cx32 which is normally expressed in hepatocytes was found to be localized in the cytoplasm of human liver tumors. Similar results were found in a human liver tumor cell line [89]. Cx43 which is not detected in normal hepatocytes was present in the invasive parts of the same tumors [31]. This means that the impaired trafficking does not depend on the type of connexin which is expressed in liver. It appears to be a general phenomenon affecting all connexins expressed in the same cells. The fact that a new connexin (Cx43) appears in the invasive parts of a tumor suggests that aberrant localization/expression of connexins may depend on the stage of the tumor. Indeed, this was shown in human prostate cancer. In this type of cancer, the connexins are localized at the cell–cell contact areas in normal and well-differentiated tumors (only Cx32 in the normal tissue and both Cx32 and Cx43 in the tumors). But progressively, the cytoplasmic localization of both connexins in the undifferentiated stages is noted [42]. In a transgenic-mouse model developing testicular tumors confined to Leydig cells, the endosomal requestration of Cx43 is an early event associated in situ with uncontrolled Leydig cell proliferation before the onset of testicular tumor invasion [90]. The cytoplasmic localization of connexins has also been observed in chemically-induced tumors suggesting it could be a general phenomenon of carcinogenesis. As an example, we can cite the cytoplasmic localization of Cx32 and Cx26 in chemically-induced rat hepatomas [58].

It is also interesting to note that the cytoplasmic localization of connexins has been associated with invasive parts of carcinomas. This is the case for Cx26 and Cx43 in chemically-induced rat bladder cancers [75]. Another example is about the apparition of Cx43 in either rat and human liver carcinomas [31,57].

3.1.2. Cytoplasmic localization of connexins in vitro

In a seminoma cell line, Cx43 was present in the trans-Golgi network [91]. But in this last case, the induced overexpression of the Cx43 was followed by the correct targeting of the connexin to the membrane and by growth decrease. In other cases, the aberrant localization of the connexins seems to be the consequence of a wrong intrinsic mechanism of membrane targeting since no induced overexpression of these proteins can change this situation. This was observed in some human colon tumor cells which express Cx43 in their cytoplasm. The transfection of a Cx43 cDNA did not improve the membrane localization of the Cx43 which was accumulating in the cytoplasm without modifying the cell–cell communication capacity tested indirectly by the lack of bystander effect [92]. The intracellular accumulation of connexins was also observed in several prostate cancer cell lines suggesting that the impaired trafficking of the connexins could be the major cause of GJIC deficiency in human prostate cancer cells [93].

The cytoplasmic localization of connexins is not always associated with abnormal or pathological situations. For instance, a transient intracytoplasmic storage of Cx43 has been described in uterine myocytes before parturition [94]. Similarly, the cytoplasmic storage of connexins represents a normal physiological process during spermatogenesis [91]. Curiously, in this last system, the storage of the connexins is associated with the presence of a 70-kDa isoform of Cx43. It has been argued that the cytoplasmic storage of Cx43 in the germ cells would play a role in cell growth control: it could allow spermatogonial proliferation at the beginning of a new wave of spermatogenesis before the recruitment of Cx43 to the plasma membrane [95]. The relationship between the localization of Cx43 and growth control is confirmed by the fact that its relocalization in the membrane is associated both with the induction of GJIC and decreased cell growth in vitro [91]. The association between Cx43 localization in the membrane and growth regulation is even reinforced by the fact that lindane induces a delocalization of Cx43 from the membrane to the cytoplasm and consequently a loss of GJIC [96]. Such a phenomenon was first observed by treating rat liver epithelial cells with lindane [97]. Apparently, lindane induces Cx43 phosphorylation and cytoplasmic localization in endosomes by activation of ERK/mitogen-activated protein kinase pathway [98].

It is interesting to note that even a nuclear localization of connexins has been reported. This is the case for Cx43 in rat liver epithelial cells transformed by either src or neu oncogenes [99]. Such a phenomenon seems to depend on the types of oncogenes which are activated since it is not observed when those cells are transformed by ras associated or not with an activated myc oncogene even if GJIC and phosphorylation of Cx43 are both decreased in all cases [99]. More recently, it was shown that the inhibition of growth of HeLa cells was induced by the carboxy-terminal part of Cx43 which was localized in the nucleus of the cells [100]. The reason for such a localization in the nucleus is not known. This may suggest that Cx43 could be involved in the control of transcription but this has not been proved yet [99]. However, without going so far, it tends to demonstrate that the formation of channels may not be always required for growth inhibition [100].

3.1.3. Mechanisms leading to the aberrant localization of connexins

The mechanisms leading to the aberrant localization of the connexins in the cytoplasm are not known. It was suggested that it is due to a cell–cell recognition impairment. Carcinomas often exhibit a decreased expression and/or aberrant localization of E-cadherin, a major transmembrane protein involved in the cell–cell recognition process of epithelial cells. Several studies have shown that the forced expression of E-cadherin in cell lines induces a more epithelial phenotype to the cells which may be accompanied by gap-junction restoration [101]. E-cadherin seems to permit a correct addressing of connexins to the cell membrane [73]. It has even been argued that such a process is the consequence of a hyperphosphorylation of the Cx43 mediated by the cadherin expression [102]. More recent data reinforce the idea of a possible involvement of cell–cell recognition in connexin localization: for instance, the induction of alpha-catenin favorizes the membrane relocalization of the connexins in human prostate cancer cell lines [93]. Such data emphasize the fact that the disturbance of GJIC would be the consequence of an aberrant cell–cell recognition process.

However, other examples do not go in such a direction. For instance, it was suggested that the cytoplasmic localization of Cx32 in human liver carcinomas could be due to a lack/decreased expression of E-cadherin but this protein is expressed in carcinomatous cells as in non-carcinomatous cells suggesting that connexin localization can be controlled by other processes [63].

3.2. Lack of heterologous gap-junctional intercellular communication

Several coculture experiments indicate that cancer or transformed cell lines had little or no GJIC capacity with their non-transformed counterparts. This was observed in different cell systems as BALB/c 3T3 cells [103], human lung carcinoma cells [34], mouse skin cells [70] and rat oesophageal cells [76]. Such an observation was even made if the tumorigenic cells do exhibit extensive intrinsic GJIC [76]. Similar results were also obtained on transformed foci which were raised from a normal cell population by oncogene transfection or chemical treatment [104]. This last approach may seem less artificial than mixing cancer cells and normal cells in the same dishes.

More convincingly, such results that could have been estimated as an in vitro artefact were also observed by using in vivo models. For instance, a selective lack of heterologous GJIC has been observed between neoplastic and surrounding normal cells by microinjecting fluorescent dye in fresh pieces of rat and human liver tissue [31,52]. It has to be noted that in both cases (rat and human), the dye-transfer assay revealed a strong reduction of the GJIC capacity compared with non-tumoral surrounding liver tissue. The lack of heterologous GJIC occurs in most of GST-P positive foci in rats [52] and is related to a decreased homologous GJIC capacity in those foci. Similarly, all human liver tumors tested by dye-transfer assay revealed a strong reduction in GJIC compared with non-tumor surrounding liver tissue [31]. In this last case, the heterologous lack of GJIC was probably due to the presence of a connective capsule around the tumors [31].

Lower GJIC capacity in adjacent tissues surrounding the tumor may be also another cause of the lack of GJIC between the cells of the tumor and their non-tumoral conterparts [31]. Such a reduction of connexin expression in the adjacent non-neoplastic tissues has also been observed in skin tumors [67].

Actually, we do not know whether such a lack of GJIC between normal and tumor cells is a common feature in human cancer. This lack of knowledge is due to the lack of sophisticated in situ approach that would permit to estimate whether GJIC does occur or not between cancer cells and normal cells in biopsies. Moreover, even if techniques would be suitable for such estimations, the lack of a clear frontline between the tumor and the normal surrounding tissues would prevent to make it. Therefore, it is impossible to estimate whether the lack of communication between tumor and non-tumor cells play a role in carcinogenesis by using in situ approaches.

We can simply hypothesize that it may play a role in growth control of cancer cells by referring to in vitro experiments in which re-induction of GJIC between the two cell types was able to prevent the growth of transformed cells [105]. This hypothesis is even reinforced by the fact that the growth inhibition of transformed cells correlates with their capacity to communicate with normal cells [106]. Such observations made GJIC suspected to be actively involved in growth control [106,107]. However, the situation is more complicated than it appears because GJIC is not obligatory required for promoting an heterologous growth control. Indeed, some studies have shown that non-transformed cells may completely suppress the growth of neighboring transformed cells without requiring gap junctions [108]. Therefore, at least in some cases, it seems that a direct intercellular contact is required for growth control even if it is not accompanied by the establishment of GJIC. We may then argue that molecules involved in direct cell–cell interaction may have such a role as they have for maintaining some cell differentiation [109].

3.3. Connexin mutation and cancer

Two different sorts of observations argued for possible mutations of connexin genes in cancer. First, the aberrant localization of connexins in cancer cells could have been the consequence of specific mutations since in vitro experiments have shown that mutations affecting connexins (which are associated with human genetic diseases) could accumulate into the cytoplasm. Secondly, several experiments have shown that connexins act as tumor suppressors which are classically mutated in cancers [6]. Consequently, the research of mutations has been performed in several types of connexins.

3.3.1. Cx37

Several studies on Cx37 mutations have been initiated from a report mentioning that mutated Cx37 is at the origin of shared

tumor-associated antigenic octa-peptides (MUT1 and MUT2) of mouse Lewis lung carcinoma cell lines (3LL and CMT cell lines) [110]. However, despite this previous result, DNA from 3LL and CMT cells did not exhibit any Cx37 mutation [111]. Since Cx37 is mostly expressed in endothelial cells, endothelial-derived tumors have been studied in order to see whether Cx37 mutations were involved in their genesis. Indeed, Cx37 mutations were detected in hepatic angiosarcomas (2 samples out of 22) from rats treated by vinylchloride. Base substitutions were detected at codon 166 (CGA to CGC) and codon 168 (GGG to GAG) in very few tumors (3/22 samples). The first mutation (3/22 samples) was silent (arginin) and the second was changing a glycine into a glutaminic acid. Cx37 proteins were detectable in endothelial cells of normal liver by immunohistochemical analysis, but none of these induced angiosarcomas showed Cx37-positive spots. These results suggest that Cx37-mediated GJIC may be disturbed in most of these angiosarcomas. However, this mutation is probably not crucial for angiosarcoma development since it was found in only one out 22 samples [112]. In addition, a silent polymorphism was detected at codon 88 [112]. In human, mutations affecting the Cx37 (proline-serine change at codon 319) were found in hemangiosarcomas. Actually, this was a polymorphism of the gene since the mutation was also found in the normal tissue of the same patients. In 84 normal donors, this polymorphism exhibited different ratios (Pro/Pro: 65.5%; Pro/Ser: 23.8%; Ser/ser: 10.7%) and, even if it does not seem to be correlated to angiosarcomas, the authors were questioning whether Ser319 predisposes to this type of cancer [113]. Another polymorphism could affect the Cx37 gene at codon 130 converting valine into isoleucine. This was found in patients suffering of breast cancer (3 tumors out of 18) and lung cancer (2 tumors out of 8) but also in the normal tissue of the same patients [114].

3.3.2. Cx32

The aberrant cytoplasmic localization of Cx32 in human hepatocellular carcinomas is not associated with any mutation in the coding region of the Cx32 gene [31]. Similar lack of mutation in Cx32 has been reported in human stomach tumors and human colon sporadic adenocarcinomas even if no study was performed about the Cx32 expression/localization in those samples [115,116]. In rats, only one chemically-induced hepatocellular carcinoma out of 12 exhibited a mutation affecting codon 220 of Cx32 [117]. This last mutation (His to Arg) was functionally silent, as tested by dye-transfer assay in HeLa cells, and responded normally to various stimuli (cAMP, 12-O-tetradecanoylphorbol-13-acetate, lysophosphatidic acid) [117].

3.3.3. Cx43

More convincing data about a possible correlation between connexin mutations and cancer concerned Cx43. Cx43 is specifically mutated (but not Cx32) in human colon sporadic adenocarcinomas [116]. All these mutations were associated to advanced stages of progression of the tumors; they were located in the carboxy-terminal part of Cx43 and led to a shift of the reading frame of the gene. Interestingly, the expression of the mutated Cx43 was restricted to the invasive structures of the tumors. It is not known yet what could be the functional consequences of such mutations on the Cx43 function and if there are really associated with the invasive phenotype of the tumors (see the part 4 of this review). This mutational phenomenon is not a general one affecting Cx43 since the lack of detectable transcripts in ovarian carcinoma cells was not the consequence of deletions or rearrangement in the Cx43 gene [38]. Similar conclusion was made for murine and human lung carcinoma cell lines exhibiting limited ability for dye-transfer and Cx43 expression [34]. No mutation of Cx43 gene was found in mouse skin tumors induced chemically [118].

3.3.4. Cx31.1

In head and neck squamous cell carcinomas a 10-fold downregulation of Cx31.1 as well as mutations in the TGF-beta-receptor-II were reported [119]. Therefore, the research of mutations affecting the Cx31.1 gene has been performed without any success meaning that no Cx31.1 mutation is involved in laryngeal tumorigenesis [119]. Only a silent polymorphism has been observed in some tumors [119].

3.3.5. Other aspects and conclusions about connexin gene mutation and cancer

Another aspect to consider is that the lack of connexin expression in cancer cells could be the consequence of mutations affecting non-coding portions of the connexin genes. Such portions are known to play a crucial role in the regulation of expression of the connexins. This is not only the case for the promoter region of the gene but also for the newly discovered IRES (Internal Ribosomal Entry Site) elements of major connexin genes such as Cx26, Cx32 and Cx43 [120–122]. Indeed, the involvement of such regions has been observed for some human genetic pathologies which are associated with altered connexin function. Most examples come from the X-linked Charcot–Marie–Tooth (CMTX) disease known to be associated with Cx32 defects [123]. If most mutations of the CMTX disease are located in the coding regions of the Cx32 gene, some were also found in the nerve-specific Cx32 promoter or in the 5′-untranslated region of the Cx32 mRNA [121,124,125]. Interestingly, the defective function or expression of Cx32 in such patients has not been shown yet to be related to a higher risk of tumorigenesis in the tissues where this connexin is normally expressed even for patients exhibiting no Cx32-coding region [126].

Previous studies on tumor-suppressor or cancer-associated genes have shown that tumorigenesis follows a two-hit mechanism that involves both gene mutations and loss of the second allele. In principle, tumor-suppressor genes include two classes: class I, in which loss of function results from mutation or deletion of DNA and class II, in which loss of function is from a block of expression. If connexins are putative tumor suppressors, they would belong to the class II which is assumed to be regulated by a different suppressor gene that lost its function by mutation or deletion [77]. This last case can be related to the altered expression of connexin-controlling transcription factors such as the hepatocyte nuclear factor 1α

(HNF-1α) that positively regulates Cx32 [127] and is often downregulated in liver tumors [128–130].

However, the reality seems to be more complex. Indeed, when only one allele of a connexin gene is mutated, it may happen that the non-functional form of the connexin encoded by the mutated allele does prevent the function of the normal one. This dominant-negative effect has been indeed observed through in vitro approaches for some connexin mutations detected in human pathologies [131]. Recent data tend to show that such dominant mutations affecting Cx26 and involved in the keratitis–ichthyosis–deafness (KID) syndrome could increase the risk of epidermal carcinogenesis [132]. This last example suggests that connexins could be also a particular class I suppressor gene for which the loss of function may result from the mutation of only one allele of the gene.

4. Connexins and metastasis

Metastasis is a complex phenomenon where cell dissociation is followed by tissue invasion, transport of metastatic cells through the blood stream, extravasation and formation of secondary tumors by colonization of foreign organs. At least in two crucial steps of this dramatic succession of events, cellular interactions are heavily involved: (1) cell dissociation leading to invasion and (2) recognition between tumor cells and endothelial cells leading to diapedesis and the formation of secondary tumors. It is probable that in these two events, gap junctions in combination with cell adhesion molecules can affect the metastatic potential. This has been hypothesized for long time but a clear picture has not yet emerged [133,134]. In this part of this review, we will consider the succession of these events only from the point of view of gap junctions.

4.1. Cell dissociation and invasion

The common hypothesis about a possible involvement of connexins in metastasis directly comes from the reduced number of gap junctions which is observed during tumor progression. There are evidences suggesting that the loss of GJIC correlates with the metastatic potential. Even if this is not always true, several models do exhibit such a correlation. This was shown in rat mammary adenocarcinoma cells [135]. The observed decrease of GJIC might be correlated to a decreased expression of the connexins. For instance, mice treated with dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) exhibit a clear reduction of Cx26 and Cx43 in the invasive sites of the induced squamous cell carcinomas [68]. Similarly, Cx43 mRNA was not detected in a highly metastatic human lung carcinoma cell line [136]. A similar observation was made in breast cancer where a correlation was found between metastatic potential and the decrease of Cx43 gene expression [137]. Restoration of GJIC has been observed in a metastatic human breast carcinoma cell line transfected with the breast metastasis suppressor 1 (BRMS1) cDNA. It increases Cx43 expression and reduces Cx32 expression, resulting in a gap-junction phenotype more similar to the normal breast tissue [137].

However, the common assumption that connexin expression is inversely correlated to metastatic potential is probably too simple. Indeed, Cx26 was found to be still expressed and even upregulated in invasive human breast carcinomas (15 samples out of 27) but the cytoplasmic localization and its heterogeneity is not compatible with an efficient establishment of GJIC [24]. The wrong localization of connexins could be the consequence of the cytoplasmic localization (or lack of expression) of cell adhesion molecules, such as E-cadherin, which is frequently observed in carcinomas. This phenomenon which is thought to be a prerequisite for cell invasion probably interferes with the gap-junction formation. This can be assumed by the accumulated evidences in the past showing that E-cadherin expression favorizes the establishment of GJIC [73,101]. It is then easy to consider that the lack of cell recognition in the primary tumor prevents the establishment of GJIC and may facilitate the invasion process.

Another aspect to consider is the cell-substrate connections. In parallel with E-cadherin lack of function, detachment from the basal membrane is often observed as a prerequisite to cell invasion. Some data suggest that these types of interactions, such as α3β1–laminin 5 interaction, could be important for maintaining GJIC by regulating the intracellular protein trafficking involved in assembly of gap junctions. This process would involve a Rho-mediated signaling [138]. According to these results, the lack of such interactions could prevent the renewal of gap-junction plaques between cells. Even if it is not well documented yet, another reason for the loss of GJIC and wrong localization of connexins could be mutations affecting connexin genes (see part 3.3 of this review). Mutations of connexins have been indeed observed in invasive portions of some tumors. This is the case for Cx43 which is mutated in invasive regions of colon adenocarcinomas [116].

Whatever the molecular events responsible for GJIC decrease are, the subsequent loss of cooperation between neighboring cells is believed to lead to cell heterogeneity and cell dissociation in the invasive parts of the primary tumor [139]. Does it mean that the invasive phenotype of tumor cells is not compatible with GJIC? Probably not since gap-junctionally coupled tumor cells can invade embryonic chicken heart fragments (ECHF), whereas non-coupled tumor cells, like HeLa cells, did not [140]. Moreover, invasion of ECHF is made possible when HeLa cells were rendered communicating after transfection of connexin cDNAs. This phenomenon seems to be independent of the establishment of heterotypic GJIC between transfected HeLa cells and chicken heart cells. If the transfected connexins (Cx31, Cx40 and Cx43) did not modify the replication rate of the HeLa cells, they were differently invasive; Cx43-expressing cells being the most invasive ones in this experimental model [141]. These in vitro data support some in vivo data in which the abnormally-augmented expression of Cx26 is responsible for the enhanced spontaneous metastasis of mouse BL6 melanoma cells. This phenotype seems to be specific to the Cx26 function since the exogenous expression of a dominant-negative form of Cx26 or the chemical inhibition of Cx26-mediated GJIC (by a oleamide derivative) prevents the spontaneous metastasis of the BL6 cells [142].

4.2. Extravasation and formation of secondary tumors

Extravasation of malignant cells often involves trans-endothelial migration (diapedesis) into tissues prior to forming secondary tumors. In contrast to diapedesis of leukocytes during inflammatory responses, little is known about the molecular mechanisms that regulate tumor–cell diapedesis.

A possible explanation could be the establishment of heterocellular GJIC between tumor cells and endothelial cells. Such a phenomenon which has been observed for breast tumor cells and endothelial cells may be an important regulatory step during metastasis [143]. This was also observed in mouse melanoma cells expressing Cx26. Increasing the Cx26 expression by transfection or inhibiting its function by a dominant-negative variant resulted as a good correlation between GJIC and metastatic capacities of the melanoma cells [144]. This observation correlates with the level of Cx26 expression which is upregulated in melanoma cells invading the dermis compared with the melanoma cells residing in the basal layer, in human samples [144]. It was concluded that Cx26 plays a role in intravasation and extravasation of tumor cells through heterologous gap junction formation with endothelial cells [144].

As a parenthesis, we see here a contradictory observation. We have seen in the previous paragraph that the decrease of GJIC could play a role in cell dissociation and invasion. Here, upregulation of Cx26 is observed in invasive parts of the human melanoma. This apparent contradiction means that the cellular event we are reviewing may be different depending on the connexin which is expressed or the cell type and the tumor type which are considered. At least, the connexin type may be important to consider since upregulation of Cx26 was observed in invasive parts of both breast cancer and melanoma in human samples [24,144]. Without going further in speculation, it is interesting to note that Cx26 upregulation is observed in some cases where cells proliferate (psoriasis, etc.).

If the establishment of gap junctions is involved in the extravasation process, it is probably just a part of a more complex phenomenon in which paracrine communication, endothelial cell adhesion and gap junctions are all involved. At least a clear interdependence has been observed between endothelial cell adhesion and communication of lung-metastatic cancer cells. It was shown that the level of coupling at focal adhesion contacts depends on sufficient amounts of Cx43 by both cell partners and, in a rate-limiting fashion, on the expression level of the receptor/ligand pair that mediates adhesion between tumor cells and the endothelium. Significantly increased adhesion and communication levels in highly lung-metastatic carcinoma cells imply a role of gap-junctional coupling in cancer metastasis, presumably by facilitating extravasation [145].

An interesting scenario describing possible molecular mechanisms involved in this complex process came from studies on HTLV-1, the human T-cell lymphotropic virus type 1, which is the causative agent of adult T-cell leukaemia/lymphoma (ATL). ATL-derived leukemic cells communicate with endothelial cells through both angiogenic-factor mediated paracrine stimulation and direct gap-junction-mediated heterocellular communication [146]. The HTLV-1 transactivator Tax seems to play an important role in this interaction by inducing the transcriptional activation of VEGF promoter and Cx43 promoter and by increasing the heterotypic communication [147]. This dual interaction between ATL-derived cells and endothelial cells induces the production of matrix metalloproteinases by endothelial cells which leads to the degradation of subendothelial basement membrane and retraction of endothelial cells, allowing then the extravasation of ATL-derived cells [147].

Local disturbance of the gap-junction pattern among endothelial cells may be also involved. For instance, it was shown that coculturing human breast cancer cells with endothelial cells leads to a rapid and transient inhibition of GJIC between the endothelial cells. Such a phenomenon is probably the consequence of interactions between the two cell types which leads to the tyrosine phosphorylation and functional inhibition of the endothelial Cx43 [148]. This local disturbance of GJIC among endothelial cells may be important since, in a model using human oral squamous cell carcinoma cells and rat lung endothelial cells, the development of cell-to-cell interactions, e.g., gap junctions and tight junctions in endothelial cells, by chemical treatment (malotilate) results in the inhibition of invasion by the tumor cells [149].

Local disturbance of GJIC between endothelial cells may be the consequence of paracrine factors produced by the tumor cells. Such a phenomenon happens during tumor–stroma interaction of skin cells: the homologous GJIC of the stromal fibroblasts is inhibited by paracrine acting factors of epithelial tumor cells. In this model, the decrease of GJIC is due to a post-translation modification of Cx43 but not to a change of expression of Cx43 [150]. This result correlates with the observation of aberrant Cx43 mRNA expression in adjacent normal lung tissue, around nodal micrometastasis of non-small cell lung cancers, which is a consequence of methylation of the Cx43 promoter [151].

Some work also suggested that gap junctions could elicit particular tissue targeting for the metastatic cells. It was said that preferential metastasis could be the consequence of the formation of heterotypic gap junctions between metastatic cells and cells of the target tissue. For instance, the formation of heterotypic gap junctions between a human breast carcinoma cell line and a human osteoblastic cell line was suggested to explain why a large extent of metastasis from breast cancers occurs in bone [152]. This was reinforced by the fact that heterotypic GJIC was even larger than homotypic GJIC between the carcinoma cells [152]. Moreover, contrary to the parental cells, transfection of a breast metastasis inhibitor, BRMS1, into the breast cancer cells increased homotypic GJIC but not heterotypic GJIC [152].

Finally, in order to close this part concerning connexins and metastasis, just a few words about the direct or undirect use of connexins as putative therapeutic tools against metastasis. It is interesting to mention that regression of established murine carcinoma metastases following vaccination was obtained with tumor-associated antigen peptides which were in fact derived from a mutated Cx37 gap-junction protein (see Section 3.3.1 of

this review) [153,154]. Moreover, the treatment of mice with a oleamide derivative able to inhibit specifically the Cx26-mediated GJIC in melanoma cells blocks partially the metastasis of these cells in mice [142].

5. Conclusions

A question which is often asked is whether the lack of connexin expression is a prerequiste for the loss of growth control. In other words, the different questions we may ask are: does the lack of expression of a specific connexin:

- induce a deregulated cell growth?
- modify the pattern of expression of genes involved in cell growth control?
- increase the susceptibility to cell transformation (at the cellular level) or carcinogenesis (at the organism scale)?

5.1. Connexin expression as a prerequisite for the loss of growth control

The recent use of transgenic animals such as connexin-KO mice did not permit in the last years to bring some clear answers to these specific questions. Indeed, if fibroblasts isolated from Cx43-null mice exhibited a higher growth rate [155], it was not the case for all cell types. For instance, primary cultures of astrocytes isolated from Cx43-null mice grow slower than their wild-type counterparts despite a lack of GJIC as tested by dye-transfer [156]. Moreover, if we presume that glial fibrillary acidic protein and S100 are good markers for estimating glial differentiation, the differentiation of the cells was not modified by the lack of the most abundant connexin of astrocytes [156]. This is in agreement with the fact that brains of Cx43-null mice are macroscopically normal and display a pattern of cortical lamination that is not detectably different from wildtype siblings [157]. It has been argued that this lack of macroscopically effect is due to the presence of a variety of other types of connexins (Cx26, Cx30, Cx40, Cx45, Cx46), detected by various techniques and at various times of culture, in those Cx43-null astrocytes [157]. Therefore, it was concluded that astrocyte gap junctions can be formed by various types of connexins and that the metabolic and ionic coupling provided by these diverse gap-junction types may functionally compensate for the absence of the major astrocyte gap-junction protein in Cx43-null mice [157]. This is actually contradictory with the fact that no dye transfer was observed in those Cx43-null astrocytes, contrary to their wild-type counterparts [156]. It is possible then that dye-transfer is not an appropriate approach to answer to this question, that more subtle GJIC mediated by compensating connexins is involved at different periods of the brain development. It is also possible that primary cultures may be an artefactual model depending on growth factors which are present in the medium.

Observing the cancer susceptibility of the whole organism lacking a specific connexin may be a better approach. A higher tumor rate has been indeed observed in such animals meaning that the lack of specific connexins may be a prerequisite for tumor formation. For instance, a higher incidence of liver neoplasms (spontaneously- or chemically-induced) was shown in mice lacking Cx32 which is the major connexin usually expressed in hepatocytes [60]. The intraperitoneal injections, two weeks after birth, of DEN led, after 1 year, both to more liver tumors in Cx32-deficient mice than in controls [60]. Since Cx32 has a stabilizing effect on Cx26, the lack of Cx32 is probably emphasized by the decrease of Cx26 in hepatocytes. Indeed, comparison of dye spreading in connexin-32-deficient versus wild-type liver revealed a 96% decrease in connexin-32-deficient tissue which would not be reached without a significant decrease of Cx26 [158].

Similarly, the deletion of one allele of a connexin gene may be sufficient to induce a higher susceptibility to tumor formation: for instance, the deletion of one allele of the Cx43 gene (and subsequent decrease of Cx43 expression) clearly favors the carcinogenic effect of urethane administration and results in a higher susceptibility to lung adenoma formation in mice [84]. In vivo, the lower Cx32 mRNA amount in female rats may also explain their higher sensitivity to liver tumors induced by hexachlorobenzene [61]. This Cx32 transcription difference between females and males seems to be controlled by ovarian hormones since ovariectomy abolished any difference between them [61]. All these examples tend to show that a decreased level of expression of a connexin may be a prerequisite for tumor growth.

5.2. The effect of a lack of connexins on gene expression

Very few studies have been performed on this topic so far. By using high-density cDNA microarrays in Cx43-null astrocytes, the analysis of gene expression revealed 4,1% of the 4998 quantifiable spots having significantly decreased hybridization compared to controls and 9,4% of the spots showing significantly higher hybridization. These different spots corresponded to RNAs encoding 252 known proteins including transcription factors, channels and transporters, cell growth and death signals, enzymes and cell adhesion molecules. These data indicate a surprisingly high degree of impact of deletion of Cx43 on others astrocytes genes: gap junction gene expression alters numerous processes in addition to intercellular communication [159,160]. Such experiments are still preliminary and it is obvious they should be extended to other types of connexins. However, those preliminary results concerning Cx43 and murine astrocytes reinforce the idea that gap junctions are involved in the regulation of gene expressions which are crucial for the cell phenotype and in particular for the control of cell growth.

Contrary to classical tumor suppressor genes which are known to control very specific molecular mechanisms, it seems that connexins do control a large panel of processes which may lead to cancer if they are not functioning properly.

5.3. General conclusions

Through all the examples which are cited in this review, we may conclude that connexins do play probably a role in

carcinogenesis. Extensive data, which have been accumulated during 40 years, suggest that it is the case. Depending on the models which have been used, it is obvious that defective gap junctions may tend to be either a prerequisite (as suggested by some studies performed on connexin-KO mice) or a consequence of the tumor development. In this last case, the decreased expression of connexins or their aberrant cellular localization can be related to some tumor progression stages. Nevertheless, whatever the deficient gap junctions are a prerequisite or a consequence of the tumor formation, they seem to give a strong impact on the development of solid tumors. As we have seen, they probably play also a crucial role in the late stages of cancer but at different levels; the invasion stage being mostly associated with a loss of function of the gap junctions, whereas a gain of function may characterize the metastasis stage.

Still, very few is known about the molecular mechanisms regulated by the gap junctions and which are responsible for growth control, invasion and metastasis. Understanding these molecular mechanisms may depend on the answer to the two fundamental and following questions:

Is such a regulation made through the establishment of GJIC? If yes, the understanding of the molecular mechanisms which are involved in growth control will depend on the identification of molecules passing through gap junctions. Such an identification is one of the biggest challenge for the researchers working in the domain of the gap junctions.

Is the cell growth regulated by connexins but independently of GJIC? If yes, what are the other functions of the connexins? Do they have these other functions through either specific interactions with particular proteins of the cytoplasm or specific localizations inside the cells? Do these other functions make them able to switch off or switch on signaling pathways involved in cell growth control?

The role of connexin is probably complex and still new theories emerge trying to bring some answers [18]. New insights concerning the direct control of gene expression by connexins are coming out, especially from connexin-transfected cells and more recently from connexin-KO mice. We may also expect interesting data coming from conditional connexin-KO mice. The induced lack of expression of a specific connexin in an adult tissue could bring interesting conclusions about the role of that connexin on cell differentiation and growth control. Such a strategy would shut down the compensation phenomenon which is observed during embryogenesis and permit the replacement of the lacking connexin by others [157]. Connexin studies should also be extended to the human situation by using primary cultures of tumor cells and not only cell lines or animal models which have their own characteristics.

Now, let us imagine for a while the domain of gap junction research as a tree. The common trunk would correspond to the discovery of gap junction, their molecular structure, the diversity of connexins as members of a multigene family, etc. From that trunk, emerging branches would characterize the accomplished progresses concerning the involvement of gap junctions/or connexins in physiological and/or pathological events. Some of these branches could eventually grow up to the full understanding of their role in such events. Newer branches of that tree have been growing quickly establishing in very recent years a clear association between certain connexins, their function and particular types of human diseases. Branches elucidating physiological roles from studies using connexin-KO mice as models were also growing very rapidly during the last decade. Paradoxically, there is not yet a so rapid growth of knowledge about the oldest branch, which emerged about 40 years ago and associates one of the most extended human diseases, cancer, with gap junctions. Over these past four decades of research, it has become clear that connexins and GJIC are involved in cancer and cellular growth control. However, there does not appear to be a single, consistent result or mechanism. This is most likely due to the diversity of connexins and gap-junction channel properties, and the cell types and context in which they are expressed. Future research will undoubtedly help to clarify these ambiguities and lead to a better understanding of the mechanisms which are involved.

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