Gap-junctions: an overlooked functional biomarker of male reproductive health

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The role of GJIC in testis

- **The critical role in:**
  - ⇒ Testicular development & homeostasis
  - ⇒ Testicular cell proliferation and differentiation
  - ⇒ Regulation of hormone production and release (testicular steroidogenesis)
  - ⇒ Initiation and maintenance of spermatogenesis

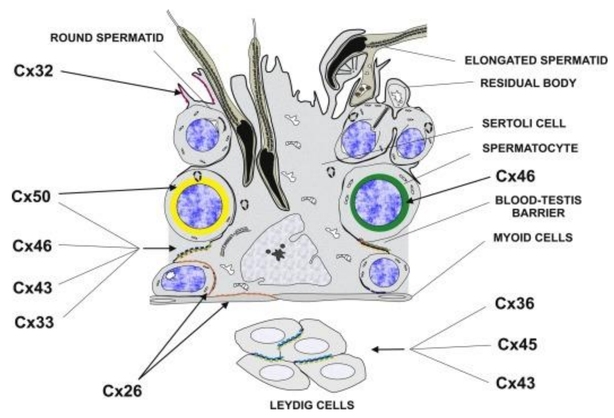
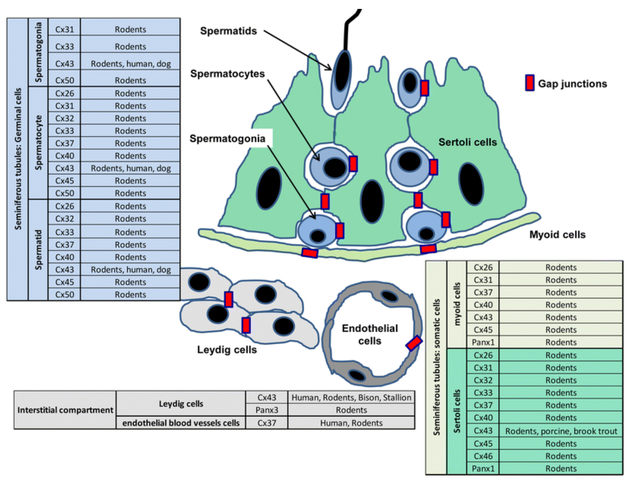


Illustration by Kidder et al. (2016) **[1]**

- **Untimely dysregulation of GJIC & Cx-related abnormalities:**
  - ⇒ Impaired spermatogenesis - increased germ cell apoptosis, spermatogonial arrest, azoospermia, germ cell deficiency
  - ⇒ Loss of blood-testis barrier integrity
  - ⇒ Hyperplasia of androgen-producing Leydig cells
  - ⇒ Leydig cell tumorigenesis
  - ⇒ Impairment of male reproductive capacity and decrease of fertility



Seminiferous tubules: Germinal cells			
Spermatogonia	Cx31	Rodents
	Cx33	Rodents
	Cx43	Rodents, human, dog
	Cx50	Rodents
Spermatocyte	Cx26	Rodents
	Cx31	Rodents
	Cx32	Rodents
	Cx33	Rodents
	Cx37	Rodents
	Cx40	Rodents
	Cx43	Rodents, human, dog
	Cx45	Rodents
	Cx50	Rodents
Spermatid	Cx26	Rodents
	Cx32	Rodents
	Cx33	Rodents
	Cx37	Rodents
	Cx40	Rodents
	Cx43	Rodents, human, dog
	Cx45	Rodents
	Cx50	Rodents

Seminiferous tubules: somatic cells		
myoid cells	Cx26	Rodents
	Cx31	Rodents
	Cx37	Rodents
	Cx40	Rodents
	Cx43	Rodents
	Cx45	Rodents
	Panx1	Rodents
Sertoli cells	Cx26	Rodents
	Cx31	Rodents
	Cx32	Rodents
	Cx33	Rodents
	Cx37	Rodents
	Cx40	Rodents
	Cx43	Rodents, porcine, brook trout
	Cx45	Rodents
	Cx46	Rodents
	Panx1	Rodents

Interstitial compartment			
	Leydig cells	Cx43	Human, Rodents, Bison, Stallion
		Panx3	Rodents
	endothelial blood vessels cells	Cx37	Human, Rodents

Illustration by Kibschull et al. (2015) **[2]**

***Cx** – connexin; **GJIC** - gap junctional intercellular communication;*

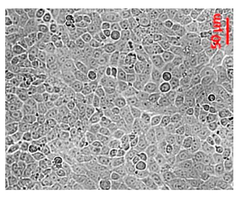
Summary & Conclusions

- Our results support the hypothesis that environmental factors are one of the major causes of male reproductive dysfunctions and that GJIC as well as connexins (Cxs) are important, but overlooked functional biomarkers of reproductive toxicants in somatic testicular cells
- Well-recognized endocrine disrupting chemical such as Vin , MTX, TCC and TCS induces a rapid dysregulation of GJIC in Leydig TM3 cells.
- EDCs can cause their reproductive toxicity in males through disturbance of junctional and/or non-junctional functions of Cx43 and through MAPK Erk1/2 and p38 signaling pathways in immature Leydig TM3 cells.
- We are now focusing on the context of cell regulatory mechanisms of GJIC with an emphasis on linking these molecular signaling events with cellular responses such as steroidogenesis, apoptosis and proliferation in 2D cultures and on the function of GJIC, Cxs and pannexins in 3D cultures of testicular cells

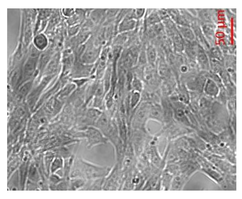
Characteristics of *in vitro* models

- **Leydig TM3** (ATCC® CRL-1714™) and **Sertoli TM4** (ATCC® CRL-1715™) cell lines ⇒ continuous, non-transformed and **non-tumorigenic cell lines** derived from immature BALB/c mouse testis **[3-7]**

TM3



TM4



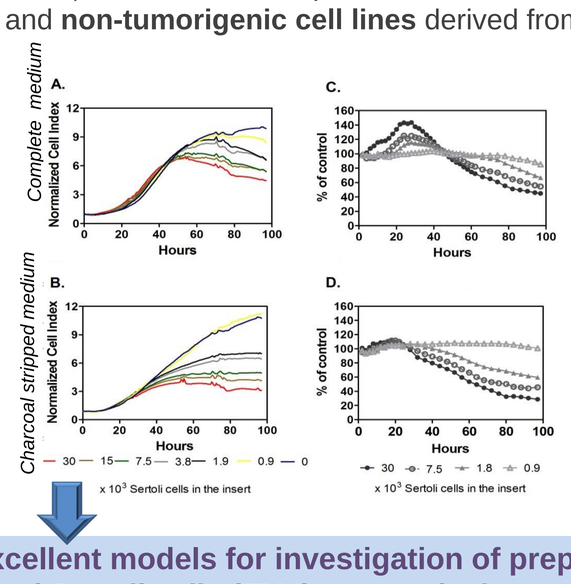
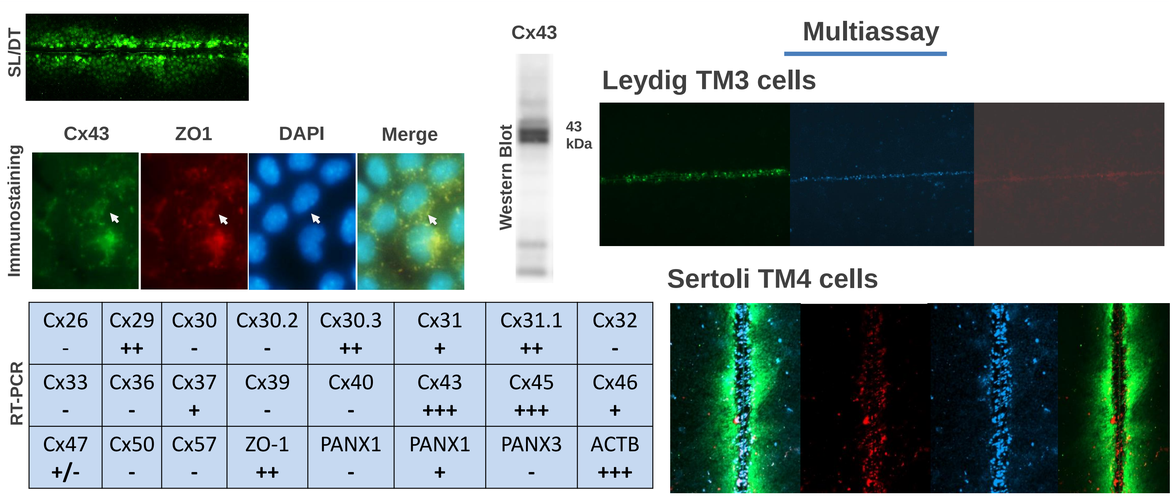


Fig. 1: Real-time monitoring of a co-culture of Leydig cells with Sertoli cells. Leydig TM3 cell on the E-Plate and Sertoli TM4 cells in the insert were cultured in medium with charcoal stripped medium (A., C.) or with complete medium (B., D.). Sertoli cells were seeded at different cell densities. For better clarity, normalized cell index was expressed (A., B.) as % of control TM3 cells growing without Sertoli cells in the insert (C., D.)

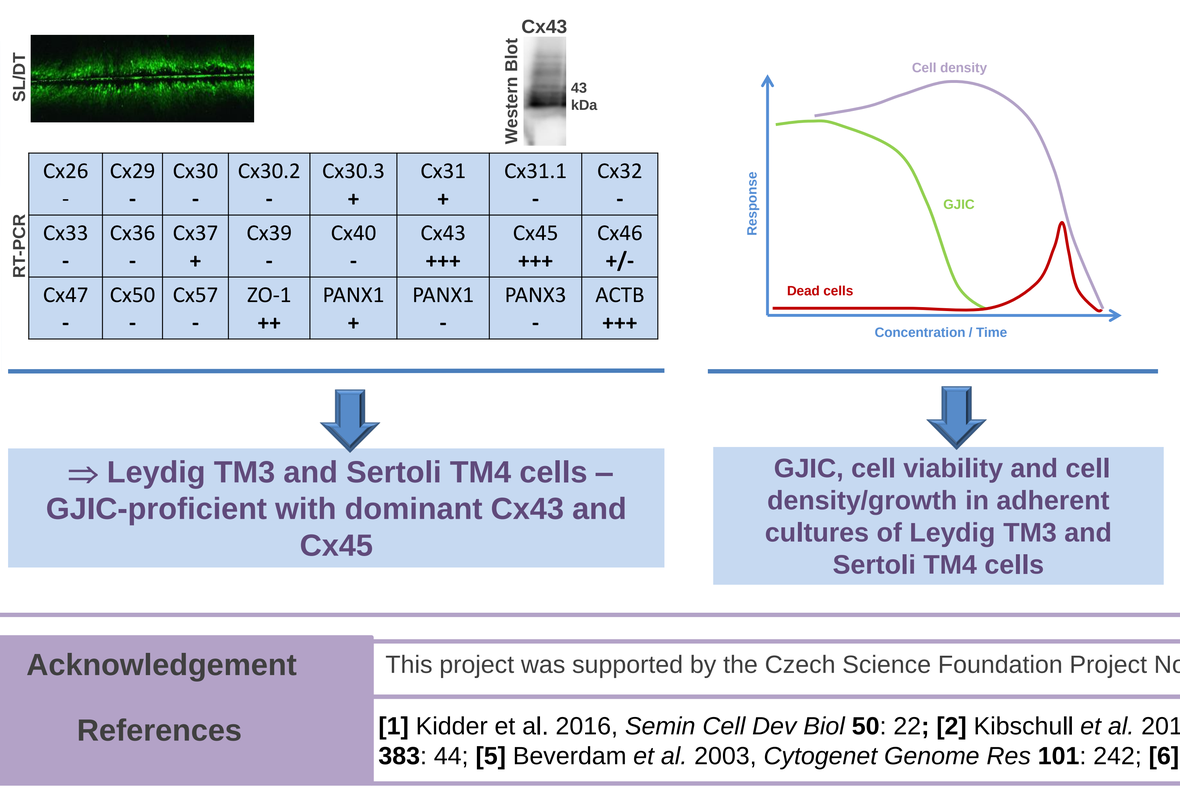
⇒ TM3 and TM4 cells – excellent models for investigation of prepubertal Leydig (TM3) and Sertoli cells (TM4), respectively

Leydig TM3 cells



RT-PCR								
Cx26 -	Cx29 ++	Cx30 -	Cx30.2 -	Cx30.3 ++	Cx31 +	Cx31.1 ++	Cx32 -
Cx33 -	Cx36 -	Cx37 +	Cx39 -	Cx40 -	Cx43 +++	Cx45 +++	Cx46 +
Cx47 +/-	Cx50 -	Cx57 -	ZO-1 ++	PANX1 -	PANX1 +	PANX3 -	ACTB +++

Sertoli TM4 cells



RT-PCR								
Cx26 -	Cx29 -	Cx30 -	Cx30.2 -	Cx30.3 +	Cx31 +	Cx31.1 -	Cx32 -
Cx33 -	Cx36 -	Cx37 +	Cx39 -	Cx40 -	Cx43 +++	Cx45 +++	Cx46 +/-
Cx47 -	Cx50 -	Cx57 -	ZO-1 ++	PANX1 +	PANX1 -	PANX3 -	ACTB +++

⇒ Leydig TM3 and Sertoli TM4 cells – GJIC-proficient with dominant Cx43 and Cx45

GJIC, cell viability and cell density/growth in adherent cultures of Leydig TM3 and Sertoli TM4 cells

Environmental contaminants target GJIC in TM3 cells

GJIC dysregulation by EDCs in Leydig (TM3) cells

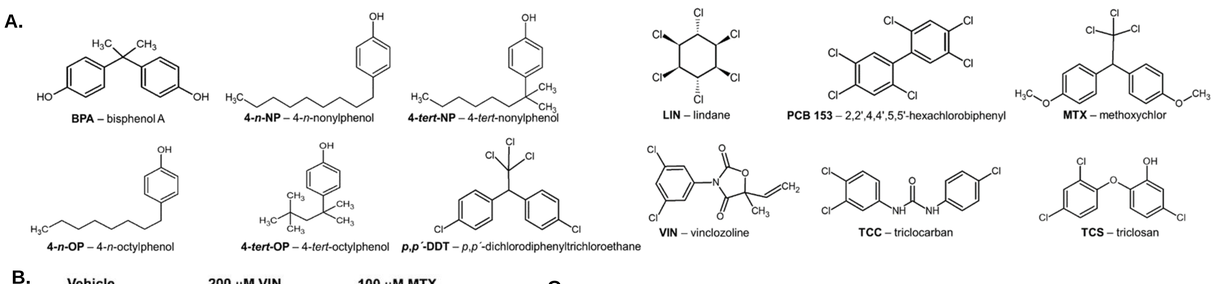


Fig. 3 The dose- and time-dependent effects of environmental contaminant **(A.)** on GJIC in Leydig TM3 **(B.)** cells treated with 6-100 μM for 0.5, 6 and 24 h. GJIC was expressed as % of communication in control cells **(C.)**

Cx43 and Cx45 expression, phosphorylation and localization in Leydig TM3

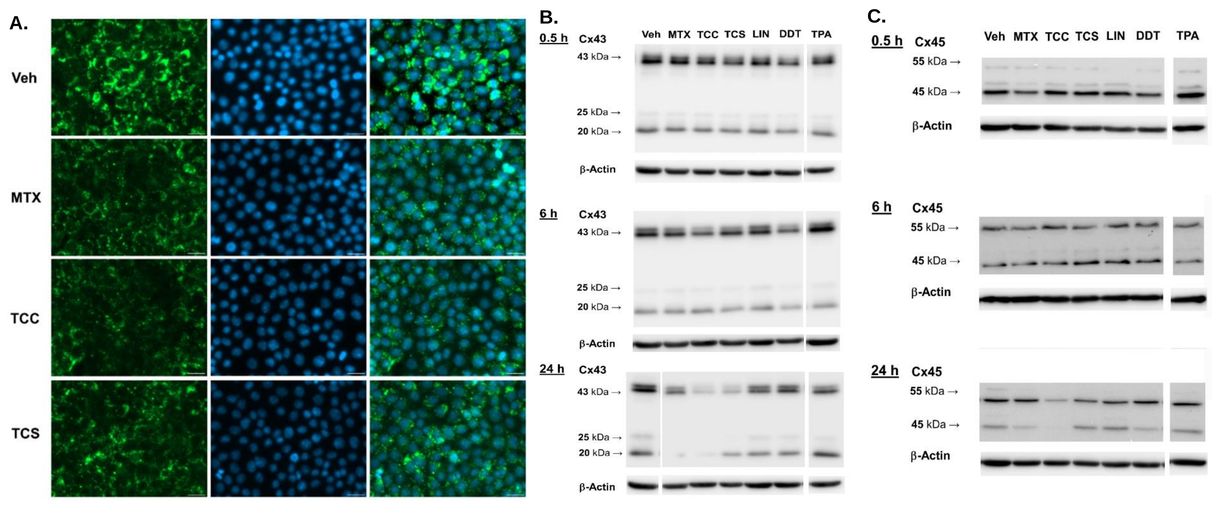


Fig.4 A decrease in immunostaining of Cx43 in the membranes of adjacent Leydig TM3 cells in response to treatment with GJIC-inhibitory **EDCs MTX, TCC and TCS (100 μM) for 0.5 h (A.),** and representative Cx43 and Cx45 immunoblots of the cells treated for 0.5 h up to 24 h (TPA: 40 nM; DDT/MTX/LIN: 100 μM; TCC 50 μM; TCS 25 μM).

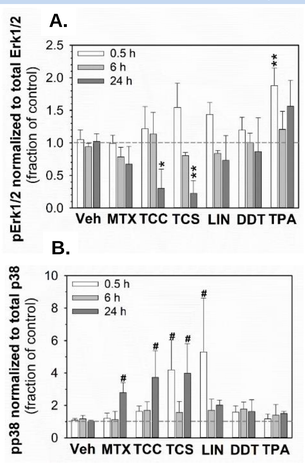


Fig.5 Phosphorylation of MAPK-Erk1/2 **(A.)** and p38 **(B.)** in Leydig TM3 cells following treatment with TPA and GJIC-inhibitory ECDs.

ECDs	Conventional Cell Viability Assay - EC_{50} (μM)						Impendence-based real-time cell analysis (RTCA) - EC_{50} (μM)																	
	AB	CFDA	NR	AB	CFDA	NR	1 h			24 h														
	1 h			24 h			0.5 h	1 h	3 h	6 h	12 h	24 h	36 h	48 h	60 h	72 h	84 h	96 h	108 h	120 h
BPA	>200	>200	>200	200	>200	>200	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
VIN	>200	>200	>200	>200	>200	>200	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
PCB 153	>100	>100	>100	>100	>100	>100	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
LIN	>200	>200	>200	>200	>200	>200	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
DDT	>200	>200	>200	>200	>200	200	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
MTX	>200	>200	>200	>200	>200	>200	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
TCC	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	65	14	10	10	11	11	12	12	13	13
TCS	83	>100	71	41	91	34	44	45	53	52	36	27	28	28	30	32	36	41	46	50
4-*n*-NP	>100	>100	>100	73	73	51	>100	>100	92	92	95	82	95	88	72	73	n.t.	n.t.	n.t.	n.t.
4-*tert*-NP	>100	>100	>100	69	93	66	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
4-*n*-OP	43	48	31	64	73	48	>100	>100	>100	>100	>100	>100	95	83	80	76	n.t.	n.t.	n.t.	n.t.
4-*tert*-OP	25	35	33	66	94	64	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

Table. 1 Cytotoxic activity of selected endocrine-disrupting chemicals (EDCs) in Leydig TM3 cells

- ⇒ All studied EDCs significantly inhibited GJIC after 0.5, 6 and 24 h of exposure with an effective concentration ranging from 13–200 μM **(Fig.3)**
- ⇒ The lowest concentrations of EDCs needed to induce rapid (within 0.5 h) significant inhibition of GJIC in Leydig TM3 cells were not lethally cytotoxic nor reducing cell viability except for TCS **(Table.1)**
- ⇒ Cx43 and Cx45 were detected as two major types of connexins in Leydig TM3 cells **(Fig.4)**
- ⇒ EDCs MTX, TCC, TCS and LIN significantly activated MAPK p38, with TCC and TCS reducing ERK1/2 activity **(Fig.5)**

Acknowledgement

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References

[1] Kidder et al. 2016, *Semin Cell Dev Biol* **50**: 22**; [2]** Kibschull *et al.* 2015, *Cell Mol Life Sci* **72**: 2879; **[3]** Mather 1980, *Biol Reprod* **23**: 243; **[4]** Mather *et al.* 1982, *Ann N Y Acad Sci* **383**: 44; **[5]** Beverdam *et al.* 2003, *Cytogenet Genome Res* **101**: 242; **[6]** Wang *et al.* 2016, Reproduction **152**: R31; **[7]** Nygaard *et al.* 2014, Sci Rep **5**: 10364.