

**Role of apoptosis and epithelial cells in *Hydra*
spermatogenesis and histocompatibility reactions**

Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Christian-Albrechts-Universität
zu Kiel

vorgelegt von

Sergey Kuznetsov

Kiel

2001

Referent/in:Prof. Dr. T. C. G. Bosch.....

Korreferent/in:

Tag der mündlichen Prüfung:

Zum Druck genehmigt: Kiel,

Teile der vorliegenden Arbeit wurden bereits veröffentlicht oder zur Publikation vorbereitet:

Kuznetsov, S., M. Lyanguzowa and T. C. G. Bosch (2001) Role of epithelial cells and programmed cell death in *Hydra* spermatogenesis. *Zoology* **104**: 25-31.

Kuznetsov, S., F. Anton-Erxleben and T.C.G. Bosch (2002) Histocompatibility reactions in *Hydra*. (In Vorbereitung)

INDEX pages

INDEX pages

1. Introduction

1.1. Programmed cell death in the animal kingdom	5
1.1.1. Cell death machinery	5
1.1.1.1. Morphological features of apoptotic cells	5
1.1.1.2. Biochemistry of apoptosis	6
1.1.2. Modulators of apoptosis	7
1.1.2.1. Survival factors	8
1.1.2.2. Anoikis: form of cell death dependent on cell-matrix adhesion	8
1.1.2.3. Quality control mediated cell death	10
1.1.3. Role of phagocytosis in apoptosis	11
1.1.4. Evolutionary aspects of apoptosis	12
1.1.4.1. Mechanisms of apoptosis are evolutionary conserved	12
1.1.4.2. Principles of apoptosis engagement in animals	13
1.2. Hydra as a model to study evolution of apoptosis	15
1.2.1. Systematics and morphology of Hydra	15
1.2.2. Tissue homeostasis and role of epithelial cells in its maintenance	19
1.2.2.1. Pattern formation	19
1.2.2.2. Cell proliferation and differentiation	19
1.2.2.3. Cell death	21
1.2.3. Cell differentiation and possible cell death in Hydra spermatogenesis	22
1.2.4. Phagocytosis and potential role of apoptosis in interspecific grafts	24
1.3. Aims of the study	25

2. Material

2.1. Animals	26
2.2. Media	27
2.3. Buffers and Solutions	27

2.4. Enzymes	29
2.5. Kits	29
2.6. Chemicals	29
2.7. Immunochemicals	31
2.8. Vectorplasmids	31
2.9. Bacteria	31
2.10. Oligonukleotides	31
2.11. Electrophoresis	32
2.12. Thermocyclers	32
2.13. Centrifuges	32
2.14. Incubators	32
2.15. UV-devices	32
2.16. Devices for histology and microscopy	32
2.17. Miscellaneous	33

3. Methods

3.1. Handling with animals	34
3.1.1. Culture condition	34
3.1.2. Grafting procedure	34
3.1.3. Preparation of heteroaggregates	34
3.1.4. Preparation of macerates	35
3.1.5. Tissue labelling with fluorescent beads	35
3.2. Histological methods	35
3.2.1. Embedding in paraffin and sectioning	36
3.2.2. Embedding in histo-resin	36
3.2.3. Electron microscopy	37
3.2.3.1. Embedding	37
3.2.3.2. Sectioning: semi-thin sections	37
3.2.3.3. Sectioning: ultrathin sections	38
3.2.3.4. Contrasting	38
3.2.3.5. Microscopy	38
3.3. Staining procedures	38

3.3.1. Histochemical procedures	38
3.3.1.1. Methylene blue / basic fuchsin	38
3.3.3.2. Feulgen staining	39
3.3.2. Immunohistochemical procedures	39
3.3.2.1. Removal of cross-species reacting antibodies	39
3.3.2.2. Immunostaining on paraffin sections	40
3.3.2.3. Immunostaining on ultrathin sections	40
3.3.2.4. Whole mount staining with antibodies	41
3.4. Apoptosis assays	42
3.4.1. Acridine orange staining	42
3.4.2. TUNEL staining	42
3.5. Molecular techniques	42
3.5.1. Extraction of genomic DNA for PCR	42
3.5.2. PCR-based methods	43
3.5.2.1. RAPD analysis	43
3.5.2.2. Amplification with sequence-specific primers	45
3.5.3. Agarose gel-electrophoresis	46
3.5.4. Cloning	46
3.5.4.1. Ligation into pGEM-T vector	46
3.5.4.2. Competent cells	47
3.5.4.3. Transformation	47
3.5.4.4. Insert-check	47
3.5.5. DNA sequencing and sequence analysis	48
3.5.5.1. Plasmid purification	49
3.5.5.2. DNA sequencing	49
3.5.5.3. Sequence analysis	49
<u>4. Results</u>	
4.1. Role of epithelial cells and apoptosis in Hydra spermatogenesis	50
4.1.1. Morphological features of Hydra spermatogenesis	51
4.1.2. Apoptosis in Hydra testes	54

4.1.3. Cell types undergoing programmed cell death	54
4.1.4. Epithelial cells act as phagocytes and remove apoptotic sperm precursors	55
4.2. Role of apoptosis in interspecific grafts in Hydra	57
4.2.1. Apoptosis is induced in interspecific grafts	57
4.2.2. Stability of interspecific grafts	58
4.2.3. Morphology of the heterotypic junction in the ectoderm	60
4.2.4. Differential epithelial movement at the graft site	62
4.2.5. The mesoglea in the chimeric region	65
4.2.6. Development of heteroaggregates	67
4.2.7. Role of histocompatibility system in induction of apoptosis	69
<u>5. Discussion</u>	
5.1. Role of epithelial cells and apoptosis in Hydra spermatogenesis	74
5.1.1. Detection of cell death during spermatogenesis	75
5.1.2. Function of programmed cell death during spermatogenesis in Hydra	75
5.1.3. Epithelial cells play an active role in Hydra spermatogenesis	78
5.2. Apoptosis in interspecific grafts of Hydra	78
5.2.1. Evidence for apoptosis in interspecific grafts	78
5.2.2. Structural incompatibility as the likeliest cause of apoptosis	78
5.2.3. Potential role of direct non-self recognition in induction of apoptosis	82
<u>6. Summary</u>	85
<u>6. Zusammenfassung</u>	86
<u>7. References</u>	87
<u>8. Appendix</u>	
8.1. Abbreviations	98
8.2. Acknowledgements	99
8.3. Curriculum vitae and publications	100

1. Introduction

1.1. Programmed cell death in the animal kingdom

Programmed cell death became one of the hottest topics in biological studies during the last decade. It was gradually recognized that cellular death in many aspects is indispensable for the life itself. Thus, it is no surprise that multicellular animals developed a specialized molecular machinery to put the death under control.

1.1.1 Cell death machinery

1.1.1.1 Morphological features of apoptotic cells

Programmed cell death was first observed during amphibian metamorphosis (Vogt, 1842) and later found to occur in many other animals during development and adult life (reviewed in Glucksmann, 1951). The term programmed cell death was initially used to emphasize that the death was somehow programmed into the developmental plan of an organism (Lockshin and Williams, 1964).

The meaning was slightly changed after Currie and colleagues studied morphology of cells undergoing programmed cell death and proposed to distinguish this type of cell death from the pathological cell death occurring accidentally at the centre of acute lesions, known as necrosis (Kerr *et al.*, 1972). They coined the term apoptosis which derives from the Greek word meaning „falling off“ of petals from flowers or leaves from trees to describe this type of cell death. The same morphological features of apoptotic cells were repeatedly observed in several tissues and proposed that they might be the result of an underlying „common, conserved, endogenous cell death programme“ (Wyllie *et al.*, 1980).

According to Kerr *et al.* (1972) structural changes in apoptosis occur in two discrete stages: the first comprises the formation of apoptotic bodies, the second their phagocytosis and degradation by other cells. The formation of apoptotic bodies involves marked condensation of both nucleus and cytoplasm, nuclear fragmentation, and separation of many membrane-bounded, compact, but

otherwise well-preserved cell remnants of greatly varying size. Apoptotic bodies are rapidly phagocytosed by other cells and undergo a process within phagosomes that is ultrastructurally similar to *in vitro* autolysis of whole cells. Apoptosis as an active and „programmed“ form of cell death is opposed to necrosis, which can be evoked by non-physiological disturbances and is thought to be a passive disintegration of a cell. Morphological features distinctive for each of these forms are summarized in Table 1.

	Apoptosis	Necrosis
Nuclear fragmentation	+	-
Chromatin condensation	+	-
Apoptotic bodies	+	-
Loss of membrane integrity	-	+
Cytoplasmic vacuolation	-	+
Mitochondrial swelling	-/+	+

Table 1. Comparison of apoptosis and necrosis by morphological criteria

1.1.1.2 Biochemistry of apoptosis

It took nearly 30 years to reveal the molecular machinery of the cellular suicide programme. It turned out that a class of proteases having a cysteine residue in the active site and cleaving substrates after aspartic acid residues play a central role in apoptosis. Therefore they were named caspases (for cysteine aspases, see Alnemri *et al.*, 1996). Their distinct substrate specificity is determined by the four residues amino-terminal to the cleavage site (Thornberry *et al.*, 1997). Caspases seem dedicated specifically to apoptosis as eliminating caspase activity will slow down or even prevent apoptosis (Earnshaw *et al.*, 1999). A biochemical definition of apoptosis emerges as a cell death with caspase activation (Blagosklonny, 2000).

Caspases bring about most of the typical morphological changes of apoptotic cells. They activate a DNase by cleaving its inhibitor, which then cuts genomic

DNA between nucleosomes, to generate DNA fragments with length corresponding to multiples of 180 - 200 base pairs (Wyllie, 1980; Nagata, 2000). Cleavage of the lamins and some other nuclear proteins by caspases is required for nuclear shrinking and budding (Rao *et al.*, 1996; Buendia *et al.*, 1999). Loss of overall cell shape is probably caused by the cleavage of cytoskeletal proteins such as fodrin and gelsolin (Kothakota *et al.*, 1997). Caspase-mediated cleavage of PAK2, a member of the p21-activated kinase family, seems to mediate the active blebbing observed in apoptotic cells (Rudel and Bokoch, 1997). Cleavage of other substrates mediates disabled DNA repair, general block of cell cycle and transcriptional activity in a condemned cell (Earnshaw *et al.*, 1999).

Caspases are constitutively expressed in all nucleated animal cells as inactive proenzymes (Weil *et al.*, 1996). They can activate each other autocatalytically. Initiator, or upstream, caspases can be activated due to intrinsic low-level proteolytic activity (Muzio *et al.*, 1998) when they get associated with activated „death receptors“ under plasma membrane, or when they associate with Apaf-1 molecule and cytochrome c, released from mitochondria, in a protein complex called apoptosome (reviewed in Hengartner, 2000)

Caspases are subject to tight regulation by different factors. Mammals and the fruit fly carry a family of genes that encodes potent caspase inhibitors, known as the inhibitors-of-apoptosis (IAP) proteins (Deveraux and Reed, 1999). Balance of pro- and antiapoptotic members of the bcl-2 family proteins control release of apoptosome components as well as other apoptosis inducing factors (AIFs) such as Smac/DIABLO from mitochondria. Smac/DIABLO promotes apoptosis through elimination of IAP inhibition (Du *et al.*, 2000).

1.1.2. Modulators of apoptosis

The suicide programme seems to be in place and ready to run in every animal cell (Weil *et al.*, 1996). Withdrawal of death suppressing signals or application of death-activating signals triggers the suicide machinery.

1.1.2.1. Survival factors

The concept of survival signalling was originally formulated by Martin Raff (1992). He suggested that most of the animal cells are ready to undergo cell death by default unless this is prevented by external signals suppressing activation of the suicide machinery. These signals can be soluble, bound to plasma membrane, or bound to the extracellular matrix. For most cells a combination of several types of signalling molecules is required for survival. It was demonstrated that a number of known growth factors function as survival factors for oligodendrocytes under cell culture conditions (Barres et al., 1992). Many types of developing neurons in mammals are thought to compete for limited amount of survival signals provided by target cells they innervate. Only about half of them get enough signal to survive while others undergo programmed cell death (reviewed in Barde, 1989). The principle of induction of apoptosis due to insufficient survival signalling is illustrated in Figure 1.1.

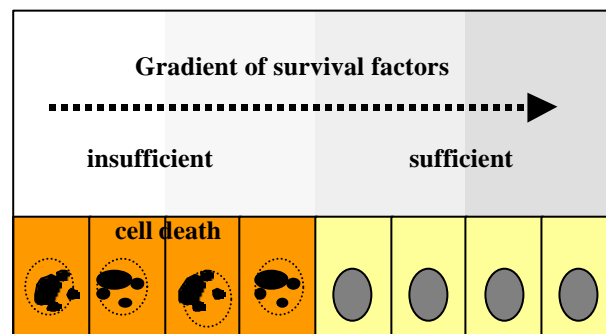


Fig. 1.1 Cell death can be triggered by the lack of survival factors

1.1.2.2. Anoikis: form of cell death dependent on cell-matrix adhesion

Interestingly, cell adhesion to the extracellular matrix (ECM) also provides a survival signal to certain cell types, first of all to epithelial cells (Meredith et al., 1993). The form of apoptosis caused by loss of adhesion to ECM is termed *anoikis*, which is the Greek word for „homelessness“ (Frisch and Francis, 1994). Importance of the anoikis mechanism is evidenced by the finding that tumour

cells frequently inactivate this pathway while becoming metastatic (Yawata et al., 1998).

Integrins are the major type of cell surface receptors mediating cell – ECM adhesion (Elangba et al., 1997). All integrins are composed of an α and β transmembrane subunits. The combination of 16 α - and 8 β -subunits allows the assembly of more than 20 different heterodimers in mammals. Depending on the subunit composition integrins bind with their extracellular domains to different ECM proteins like fibronectin, laminin and collagen thereby forming an effective cell-matrix association. A number of different proteins are associated with intracellular portion of integrins forming a cytoplasmic plaque. Some of them mediate anchorage of cytoskeleton to cell-matrix adhesion sites. Others mediate integrin-specific signalling (reviewed in Ruoslahti, 1996).

How integrins bound to ECM confer resistance to apoptosis is still far from being understood. Nevertheless a few molecules such as the focal adhesion kinase (FAK), Shc and integrin-linked kinase (ILK) were linked to anoikis (reviewed in Frisch and Screatton, 2001). These molecules activated by integrin can mediate enhanced expression of antiapoptotic bcl-2 family members. FAK was demonstrated to directly regulate the expression of the IAP family of caspase inhibitors. All of them might also potentially converge on other known antiapoptotic signalling pathways (Frisch and Screatton, 2001).

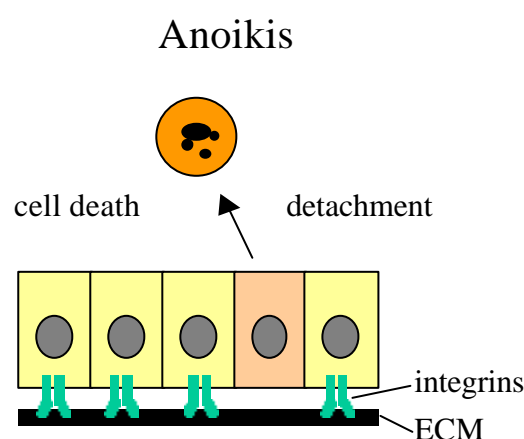


Fig. 1.2 Model of anoikis

1.1.2.3. Quality control mediated cell death

Programmed cell death also functions as part of a quality control process, eliminating cells that are abnormal, misplaced, nonfunctional, or potentially dangerous to the organism (Jacobson et al., 1997). For instance, in vertebrate immune system T and B lymphocytes that either fail to produce functional antigen receptors or bear self-reacting receptors are eliminated by apoptosis via specific death receptor pathway (reviewed in Krammer, 2000).

Apoptotic programme can be triggered in response to stress factors such as DNA damage (Lowe et al., 1993). DNA damage, especially double strand breaks, can cause serious mutations and lead to malignant transformation of a damaged cell that would be deleterious for the whole organism (Hanahan and Weinberg, 2000). Several mechanisms are immediately activated in such cases to block progression through the cell cycle and to repair the DNA lesion. When the lesion turns out to be irreparable, the cell commits suicide for the sake of the entire organism (reviewed in Rich et al., 2000).

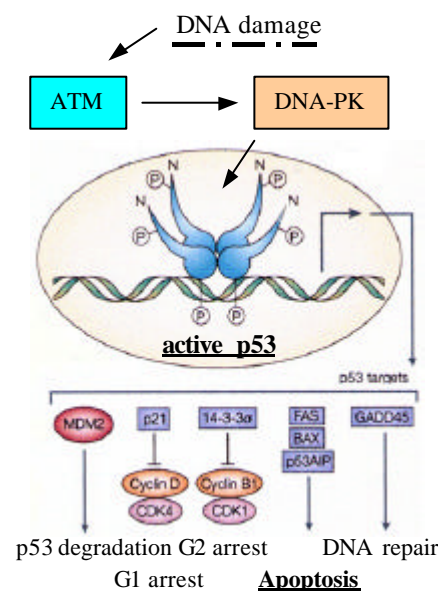


Fig. 1.3 p53-mediated quality control of cell death after DNA damage (From Bullock and Fersht, 2001)

Tumour-suppressor protein p53 plays a crucial role in mediating cell death after DNA damage. This protein is a transcription factor. Being activated by DNA damage sensor molecules, p53 can initiate transcription of negative regulators of cell cycle, proteins promoting apoptosis as well as proteins mediating DNA repair. Thus, activation of p53 does not necessarily mean that apoptotic cell death will follow. Whether a damaged cell undergoes apoptosis or survives, is most probably a question of the balance between pro- and antiapoptotic signals in it (Rich et al., 2000).

1.1.3. Role of phagocytosis in apoptosis

As Currie and colleagues noticed in their seminal paper (Kerr et al., 1972), free apoptotic cells are rare to observe in a tissue. Most of them can be found inside of phagocytic vacuoles of neighbouring cells. They proposed that phagocytosis and subsequent degradation of apoptotic bodies represent a second stage of the cellular suicide programme. It is important that phagocytosis of apoptotic bodies occurs before integrity of their membrane is lost. This prevents leakage of their contents into surrounding tissues and provoking of inflammatory responses (Savill et al., 1989). Recognition and engulfment of apoptotic cells appears to occur very quickly. In the nematode *Caenorhabditis elegans*, where the cells sentenced to die are predetermined very early during development, engulfment can begin even before the completion of the cell division that generates a doomed cell (Robertson and Thomson, 1982).

For phagocytosis to occur, apoptotic cells have to expose „eat me“ signals on their surface. In addition, phagocytes must possess appropriate recognition and signalling instruments to induce formation of cellular protrusions engulfing cell corpses. Some of these mechanisms have been elucidated during the last decade. It turned out that in apoptotic cells the ATP-binding cassette transporter 1 (ABC1) becomes activated. This huge protein regulates membrane-lipid turnover and cholesterol exchange, and leads to exposure of phosphatidylserine, normally restricted to the inner-membrane leaflet, on the surface of the apoptotic cell, thus representing an „eat me“ flag (Hamon et al., 2000). Recently a specific

phosphatidylserine receptor expressed on the surface of macrophages, fibroblasts and epithelial cells has been identified, thus completing one of the recognition pathways (Fadok et al., 2000). Other molecules such as specific lectins, integrins (different from those used in cell-matrix adhesion), and scavenger receptors as well as trombospondin and C1q component of complement, functioning as „bridging“ molecules, have also been implicated in corpse recognition by phagocytes (reviewed in Platt et al., 1998; Savill and Fadok, 2000). Whatever the receptors are, they activate a signalling complex comprising adaptor molecules CrkII and DOCK 180, and a small GTPase Rac1, which confer necessary for engulfment cytoskeletal changes in phagocytes.

Recently two groups have simultaneously found that in the nematode *C. elegans* engulfment genes cooperate with caspases and thereby promote apoptosis (Hoeppner et al., 2001; Reddien et al., 2001). Mutual interaction between cells undergoing apoptosis and phagocytes could also take place in mammals and other animals, as both processes are very conserved throughout the animal kingdom.

1.1.4. Evolutionary aspects of apoptosis

1.1.4.1. Mechanisms of apoptosis are evolutionary conserved

The apoptotic machinery is conserved throughout the animal kingdom. Homologues of essential regulators of apoptosis such as caspases, Apaf-1, pro- and antiapoptotic bcl-2 family members, and p53 as well as molecules containing death domain were found in the nematode, fruit fly and mammals (reviewed in Meier et al., 2000). The strongest evidence of their conservation is the fact that human Bcl-2 is fully functional in suppressing cell deaths in developing *C. elegans* instead of CED-9 (Vaux et al., 1992). Caspases, proteins dedicated to apoptosis, were recently found even in the cnidarian polyp *Hydra* (Cikala et al., 1999). However, the number of apoptotic regulators and their redundancy mirrors the complexity of the animal organization. Thus, in vertebrates at least 15 caspases were identified, in the fruit fly *Drosophila melanogaster* 5 identified and another 2 predicted, in the nematode *C. elegans* 3 and in the cnidarian *Hydra vulgaris* 2 caspases were found (Meier et al., 2000; Cikala et al., 1999).

No caspases were found, however, outside of the animal kingdom. Nevertheless, two families of caspase-like proteins, paracaspases (found in metazoans and slime mold *Dictyostelium*) and metacaspases (found in plants, fungi, and protozoa), were identified recently (Uren et al., 2000). *Dictyostelium discoideum* and plants were also reported to undergo programmed cell death. Programmed cell death in plants shows features similar to metazoan apoptosis such as internucleosomal DNA fragmentation, nuclear fragmentation and even involvement of caspase-like activity (De Jong et al., 2000; Korthout et al., 2000), though this does not seem to be the case in *Dictyostelium* (Olie et al., 1998). A growing body of evidence suggests the existence of other, caspase-independent, pathways of programmed cell death in animals morphologically appearing similar to necrosis (Kitanaka and Kuchino, 1999). It is a matter of debate how similar are cell death programmes between animals and other living organisms and how such a complex system could arise during animal evolution.

1.1.4.1. Principles of apoptosis engagement in animals

Apoptosis plays an important role in animals both during development and in their adult life (schematically illustrated in Figure 1.4). It is widely exploited to sculpture tissues and organs, e.g. apoptotic death of interdigit cells during vertebrate limb development (Montero et al., 2001) or hollowing out solid structures to create lumina (Coucovanis and Martin, 1995). Larval structures are removed by apoptosis during metamorphosis as it happens to the amphibian tadpole tail (e.g. Sachs et al., 1997). Primordial structures not needed for an adult organism also can be removed by apoptosis, e.g. Müllerian duct in male and Wolffian duct in female mammals, respectively. Anoikis provides a simple and genuine way to build a single-layered epithelium out of an unordered cluster of cells, whereby only cells having a direct contact to the basement membrane would survive. Apoptosis mediated by death signals is widely used in immune system to eliminate dangerous and infected cells. Finally, cell death is an ultimate measure against mutated cells getting out of organismal control (reviewed in Jacobson et al., 1997; Meier et al., 2000).

It appears that the programme controlling cell death arose nearly simultaneously with multicellular animals. How it happened and what kind of selection pressure

facilitated its evolution is not clear. Luis Wolpert (personal communication, evening lecture at the meeting on cnidarian development, Tutzing, Germany, 2001) has speculated that prototypic multicellular organisms might retain daughter cells after cell divisions and adopt controllable killing of some cells to provide food to remaining ones under unfavourable environmental conditions. Soon quick and effective cell death proved useful for many other needs of metazoa. According to another hypothesis the key advantage of apoptosis over necrosis is that apoptotic cells when being phagocytosed do not elicit inflammatory response in contrast to necrotic cells (Savill and Fadock, 2000). However, this argument is hardly applicable for evolutionary oldest animals, which have no circulatory system and no specialized immune system.

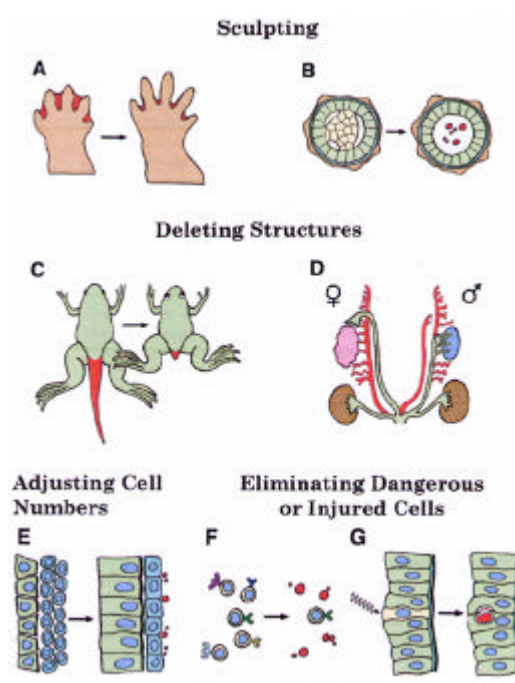


Fig. 1.4 Some functions of programmed cell death in animal development
(From Jacobson et al., 1997)

To get insight into the early steps of apoptosis evolution, it seems sensible to study apoptosis in the most basal organisms known to possess components of the apoptotic machinery.

1.2. Hydra as a model to study evolution of apoptosis

1.2.1. Systematics and morphology of *Hydra*

Hydra belongs to one of the most basal eumetazoan phylum *Cnidaria*, class *Hydrozoa*.

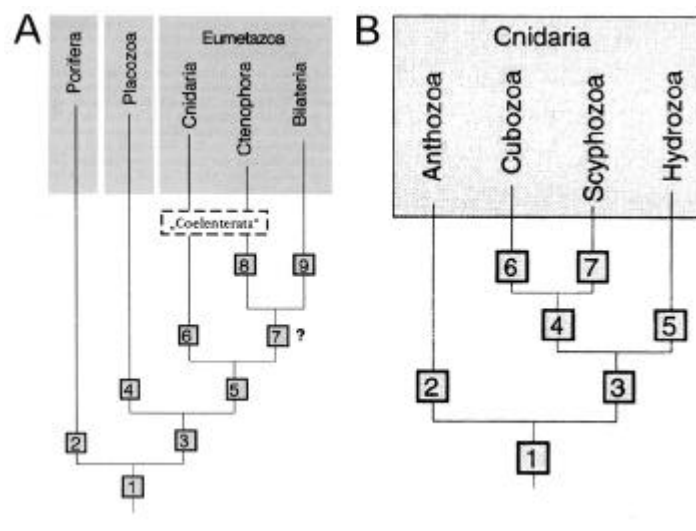


Figure 1.5. Systematics of Cnidaria. (A) Position of Cnidaria among metazoa. Apomorphic traits are as follows: 1) Oocytes with polar bodies; spermatozoa with a head and a tail; fibrillar extracellular matrix; 2) Pinacoderm, choanoderm and intervening mesohyll; 3) Tight junctions, gland cells; 4) Covering layer is differentiated to upper- and lower sides; the latter is specialized for food uptake; 5) Two germ layers giving rise to true epithelia, epidermis and gastrodermis, the latter lining up a gastric lumen; body opening functioning as a mouth and anus; sensory cells and neurons; gap junctions with connexins; 6) Cnidocysts in cnidocytes (nematocytes); asexually propagating polyps; 7?) acrosome with an acrosomal vesicle and a subacrosomal structure beneath; 8) A pair of tentacles and colloblasts; bilateral symmetry; complex apical vertex with a statocyst; 9) Triploblastic organization with endomesoderm; bilateral symmetry with anterioposterior polarity; anterior brain; specialized excretion organs. **(B)** Class-level relationships within the phylum Cnidaria. Apomorphic traits are as follows: 1) Nematocysts; nematocytes with a flagellum; planula-larvae; polyp stage; 2) octaradial gastric cavity; bias to bilateral symmetry; 3) Nematocytes with a modified flagellum (cnidocilium); microbasal euryteles; podocysts; linear mtDNA; 4) polyp tentacles without endodermal cavity; medusas with rhopalia; 5) most developed cnidom; ectodermal gonads; 6) medusas formation through metamorphosis of a polyp; medusas with pedalia and velarium; 7) strobilation. (From Westheide and Rieger, 1996).

Hydra is a small freshwater polyp about 1.0 cm in length. Its radially symmetric body with only one axis represents practically a simple tube with two differentiated structures at both ends (Fig. 1.6). Hypostome with a mouth opening and a swirl of tentacles terminates its distal (apical) end. Foot with a basal disc terminates the proximal (basal) end of the body. *Hydra* is a sedentary animal. Specialized cells at the basal disc secrete mucous glue to attach the animal to the substratum (Hoffmeister and Schaller, 1985). Most of the *Hydra* body column is occupied by the gastric region as it contains the gastric cavity where digestion takes place. In the lower one third of the body column budding region can be identified where new polyps grow out. Budding is the main mode of propagation in *Hydra*. In contrast to most other hydrozoans, *Hydra* lacks medusoid generation, which usually bears the function of sexual reproduction. Gonads occasionally differentiate in *Hydra* in the ectoderm (see below).

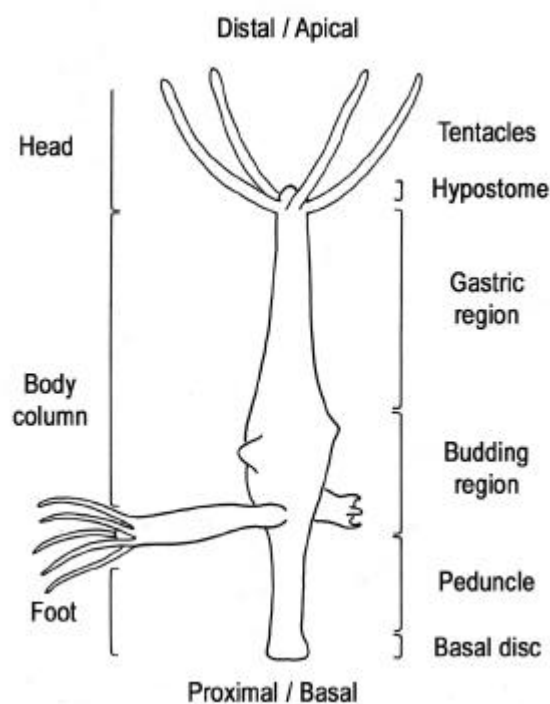


Figure 1.6. Morphology of a *Hydra* polyp. (From Holstein, 1995)

The basal phylogenetic position of *Hydra* among other metazoa is reflected in its histological organization (Figure 1.7). As characteristic for diploblastic animals, the body wall of a *Hydra* polyp is built of only two epithelial layers, ectoderm and endoderm. Ectodermal and endodermal epithelial cells build true single-layered epithelia. Adjacent epithelial cells are sealed together by narrow bands of septate

junctions analogous to tight junctions in vertebrates just beneath their apical surface (Wood, 1959). Septate junctions divide apical and basolateral surfaces of epithelial cells, thus, establishing epithelial polarity. They also isolate the inner milieu of the body from the outer world. Ectodermal epithelial cells secrete glycocalyx, a carbohydrate coat, at their apical surface. Little is known about its function in *Hydra*. At the basal surface epithelial cells of both ectoderm and endoderm lay out a thin acellular layer of mesoglea, intervening between both epithelia (Holmes, 1950). Mesoglea composition is similar to that of extracellular matrix of vertebrate animals (Figure 1.8). The central fibrous zone contains type I collagen, heparan sulfate proteoglycan and fibronectin-like molecules (Sarras et al., 1991). Laminin and probably type IV collagen constitute basement membrane in subepithelial zone of mesoglea (Day and Lenhoff, 1981; Sarras et al., 1994). Epithelial cells of both layers have two muscular protrusions mediating body contraction and dilation (Mueller, 1950). Endodermal epithelial cells function mainly as digestive cells. Extracellular digestion is facilitated by respective enzymes secreted by gland cells into the gastric cavity.

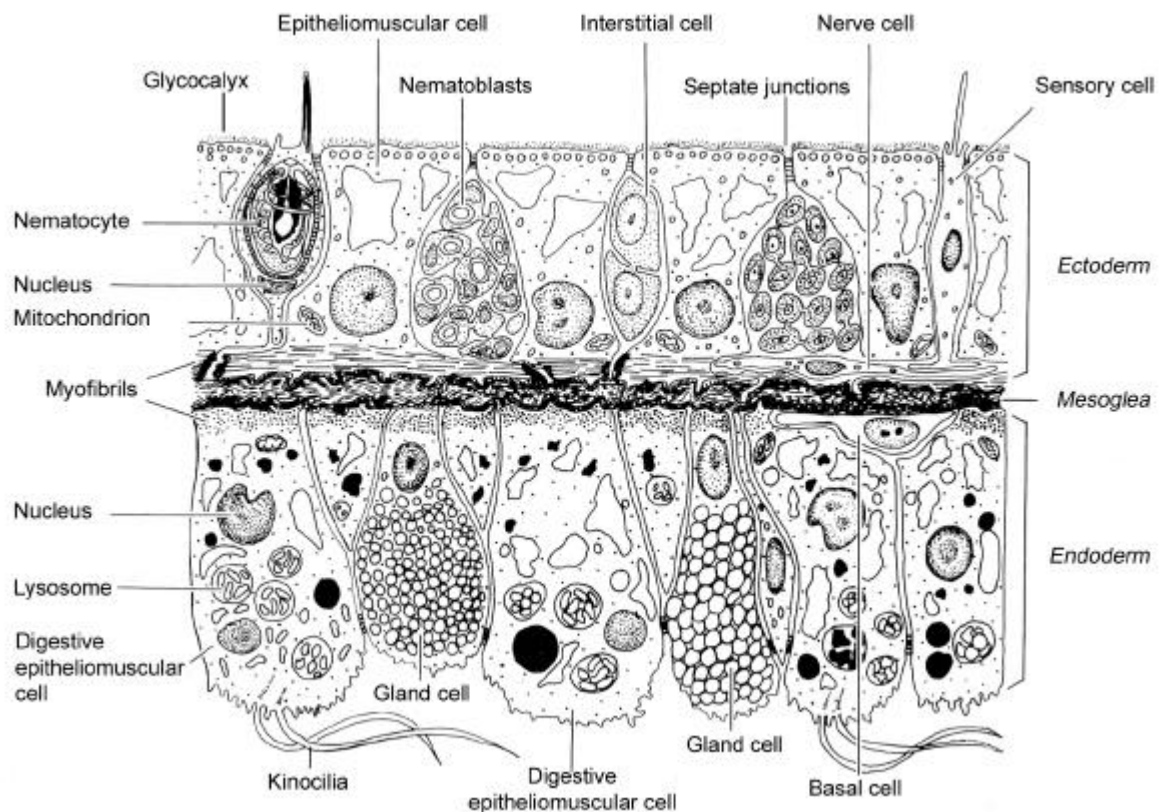


Figure 1.7. Histology of *Hydra*. (from Tardent, 1978)

Both epithelia contain nerve cells. *Hydra* nervous system is considered to be one of the most simply organized in the whole animal kingdom. It appears as a diffuse plexus of nerve cells spread over the whole polyp body (Schneider, 1890). Higher density of the neuronal network can be observed in hypostome and a basal disc. Sensory, ganglion and motor neurons can be identified (Davis et al., 1968). Hidden complexity of *Hydra* neurons was recently revealed as distinct neurons may secrete different neuropeptides (Hansen et al., 2000).

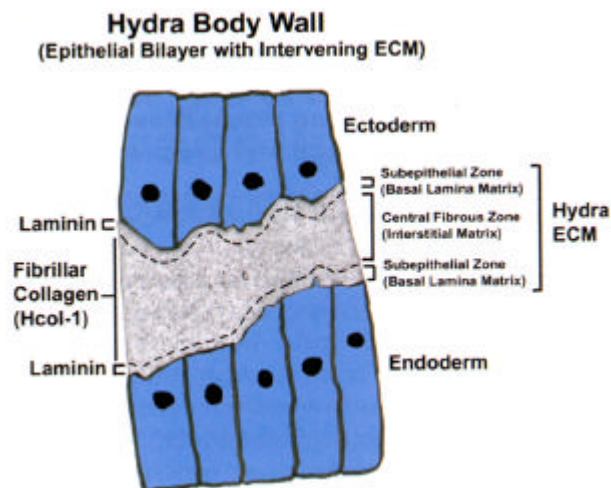


Figure 1.8. Hydra mesoglea. (From Sarras, 2001)

Cnidaria-specific cell types, nematocytes or stinging cells, are located in the ectoderm. *Hydra* has four distinct types of nematocytes. They differ by the type of secretory vacuole, the capsular nematocyst, appearing like an inverted coiled tube, which can eject its content in a discharge process (Holstein, 1981). Nematocytes mediate penetration and capture of a prey. They are most concentrated in tentacles where they are embedded into epithelial cells, here called battery cell. One battery cell contains about 8-24 nematocytes (Campbell, 1987).

Interstitial cells (i-cells) are located in intercellular spaces of epithelial cells and comprise up to 75% of all cells in the animal (David et al., 1987). These are cells with relatively large nucleus and a narrow rim of cytoplasm. They function as multipotent stem cells able to differentiate into nematocytes, gland cells, neurons and germ line cells (David and Murphy, 1977; Schmidt and David, 1986; Bosch and David, 1987).

1.2.2. Tissue homeostasis and role of epithelial cells in its maintenance

1.2.2.1. Pattern formation

Hydra polyp has a defined body plan or pattern, reproducible from one polyp to another, which has to be maintained. *Hydra* is also famous for its enormous regenerative capacities noticed more than two centuries ago (Trembley, 1744). When a head or a foot is cut, the polyp builds a missing body part anew by repatterning of existing tissues without much new growth, a process known as morphallaxis (Gilbert, 2000). A new polyp can regenerate even from a disordered clump of cells produced by reaggregation of cells out of a single-cell suspension (Gierer et al., 1972).

Combined efforts of several labs revealed several of molecules participating in defining specific structures of a *Hydra* body such as the foot, hypostome, tentacles, and the single body axis (Technau and Bode, 1999; Hobmayer et al., 2000; Gauchat et al., 2000; Lohmann and Bosch, 2000). Surprisingly, albeit *Hydra* body plan differs greatly from that of higher animals, some of its key morphogens are homologous to those in the fruit fly and amphibians (Hobmayer et al., 2000; Arendt et al., 2001). Mathematical models have been produced describing the basic principles of patterning in an animal like *Hydra* (Gierer, 1977; Meinhardt and Gierer, 2000). Studies are now under way to bring the theory and praxis together.

Epithelial cells play a crucial role in pattern formation. This is evidenced by the fact that *Hydra magnipapillata* sf-1 polyps, harbouring temperature-sensitive i-cells and lacking all cell types except for epithelial cells after heat treatment (so-called epithelial *Hydra*), are able to maintain the body pattern and regenerate missing structures (Sugiyama and Fujisawa, 1978). This is consistent with epithelial localization of most of the molecules implemented in pattern formation.

1.2.2.2. Cell proliferation and differentiation

Approximately 10^5 cells split in only several distinct cell types and comprise a single adult *Hydra* polyp (Bode et al., 1973). All the cell types can be assigned to one of three self-renewing cell populations. While both ectodermal and

endodermal epithelial cells retain the capacity to occasional cell division, interstitial stem cells reproduce themselves but eventually undergo terminal differentiation in one of the four possible directions: nerve cells, nematocytes, gland cells or germ cells (David et al., 1987). The final decision is influenced by already present terminally differentiated cells of a certain cell type as well as by other cell types. For instance, neuron differentiation is negatively influenced by a short Hym-33H peptide secreted by ectodermal epithelial cells and positively by Hym-355 peptide produced by a subpopulation of ganglion cells (Bosch and Fujisawa, 2001). Little is known about regulation of differentiation of other cell types.

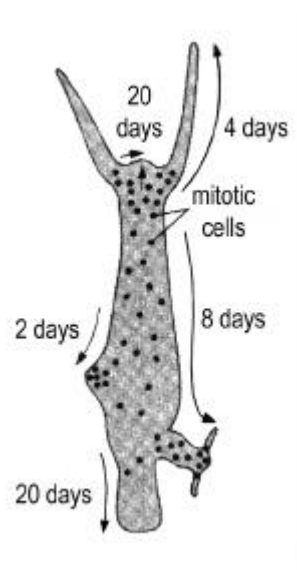


Figure 1.9. Cell flux in *Hydra*. Arrows indicate directions in which cells are displaced after a certain number of days (From Müller, 1995).

Hydra tissues are constantly in flux. Single interstitial cells and nematocytes can actively migrate using fibronectin of extracellular matrix as a substrate (Stidwill and Christen, 1998). Epithelia migrate as sheets rather than as single cells and movement of ectodermal layer may be not very tightly coordinated with that of the endodermal epithelium, sometimes depending on a species (Campbell, 1974). As a result of cell proliferation in the gastric region most cells are passively displaced distally into tentacles and hypostome or proximally into growing buds and foot as illustrated in figure 1.9. Cell proliferation in *Hydra* can be modulated by feeding conditions or morphogenetic processes (Holstein et al., 1991). Thus, it is even

more surprising that under starvation conditions cell proliferation does not stop in *Hydra*. Instead, new cells continue to occur while using own phagocytosed cells as food supply what results in gradual decrease in size of the polyp (Bosch and David, 1984).

1.2.2.3. Cell death

As most other metazoa *Hydra* possesses a machinery for the programmed cell death. Two caspases, the key regulators of apoptosis, have been identified recently in *Hydra vulgaris* (Cikala et al., 1999). Sequence analysis revealed that they are most closely related either to effector caspases-3 of vertebrates or to ced-3 caspase of the nematode *C. elegans* (see figure 1.10.). The authors also shown that *Hydra* caspase-like activity could be readily blocked by caspase-3-specific and to a lesser extent by caspase-1-specific inhibitors. Other characteristic features of cells undergoing programmed cell death such as chromatin condensation, nucleus fragmentation and DNA laddering were demonstrated (Cikala et al., 1999). Thus *Hydra* represents the lowest multicellular animal having apoptotic machinery known to date.

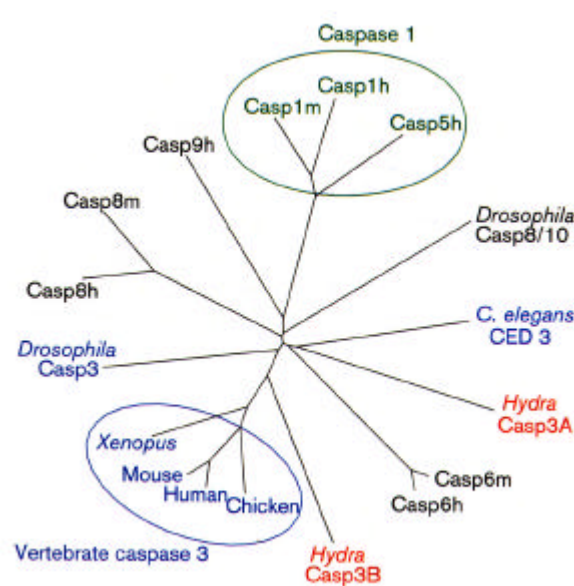


Figure 1.10. Phylogenetic comparison of hydra caspases with caspases from other animals (from Cikala et al., 1999).

In *Hydra* cell death opposes cell proliferation. While cells proliferate mostly in the gastric region and get displaced to the both ends of the *Hydra* body, they die by apoptosis in the hypostome, tentacles and in the foot (Lyon et al., 1982; Cikalá et al., 1999). Epithelial cells play an important role in *Hydra* as non-professional phagocytes by removing dead cells (Campbell, 1976; Lyon et al., 1982). Phagocytic activity of epithelial cells is probably essential for *Hydra* as a means to reutilise dead material like it happens under starvation conditions (Bosch and David, 1984).

1.2.3. Cell differentiation and possible cell death in *Hydra* spermatogenesis

Although budding is the primary mode of propagation, all *Hydra* species have been observed to go through occasional sexual phases. Onset of egg and sperm differentiation is triggered by environmental signals. Under laboratory conditions, sexual differentiation of *Hydra* is induced by low temperatures or starvation (Littlefield et al., 1991). There are no pre-formed structures where gonads would appear. Testes form as conical ectodermal swellings usually in the upper gastric region while ovaries appear in the lower gastric region in a form of so-called egg flecks (Figure 1.11. A).

Germ cells in *Hydra* derive from multipotent interstitial stem cells (Bosch and David, 1986), which aggregate in the intraepithelial space to form ovaries and testes (Tardent, 1985). Spermatogenesis is initiated by local accumulations of interstitial cells within the intercellular spaces of ectodermal epithelial cells. As these clusters of spermatogonia grow they lift the stratum of the epithelial cells, which then forms the protruding outer wall of the testis (Tardent, 1974). Between the mesoglea and the outer epithelial wall, the various cell layers represent the succession of the various stages of spermatogenesis (Figure 1.11 B; Brien, 1966). Clusters of accumulated interstitial cells synchronously undergo meiotic divisions and the subsequent process of spermiogenesis (Munck and David, 1985). The mature spermatozoa, which already move in the testis, are released into the surrounding medium in small portions of 10 to 100 at a time. In a single testis 3 to 5 such subsequent ejaculations are normally followed by a period of

rest lasting several hours (Zieler, 1972). A single testis stays active for 7-8 days followed by resorption (Brien and Reniers-Decoen, 1951).

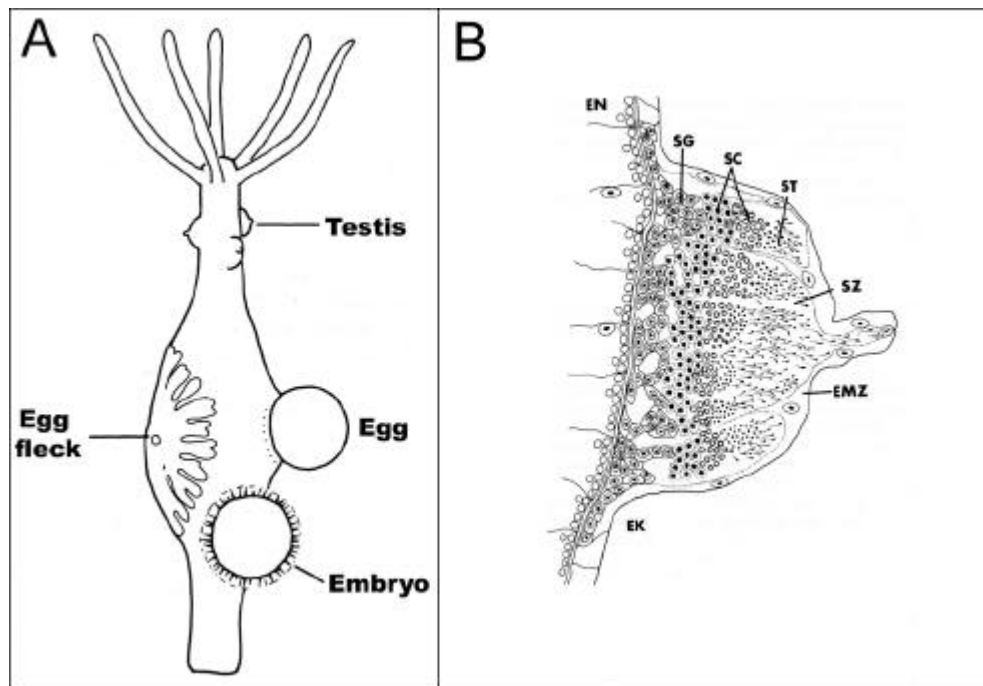


Figure 1.11. Gametogenesis in Hydra. (A) Morphology and location of male and female gonads. **(B)** Spermatogenesis. SG – spermatogonia, SC – spermatocytes I and II, ST – spermatids, SZ – spermatozoa (sperm), EMC – epitheliomuscular cell, EC – ectoderm, EN – endoderm (From Holstein, 1995).

While the differentiation pathway as well as the structure of the mature spermatozoa is very similar in *Hydra* and higher metazoa, nothing is known about the temporal and spatial signals controlling spermatogenesis. In higher animals germ cell proliferation and differentiation is regulated by a complex network of endocrine and paracrine signals. Programmed cell death is involved in maintaining homeostasis of each germ cell type during spermatogenesis and quality control of sperm precursors (reviewed in Print and Loveland, 2000). One can expect that *Hydra* also might use such mechanisms to control spermatogenesis.

1.2.4. Phagocytosis and potential role of apoptosis in interspecific grafts

Apoptosis plays an important role during development of vertebrate immune system and in execution of its functions as described in chapter 1.1. *Cnidaria* have no specialized immune cells. However, as shown on the colonial hydroid *Hydractinia*, they possess a genetically determined system to discriminate between „self“ and „non-self“ in terms of allorecognition (Mocady and Buss, 1996). *Hydra* was also suggested to have a similar system (Bosch and David, 1986). This was tested in a model situation of interspecific grafts between polyps of two easily distinguishable species, *H. vulgaris* and *H. oligactis* (see Materials and Methods). When halves from different species were grafted together as illustrated in figure 1.12. A, one species apparently eliminated foreign tissues in a species-specific manner.

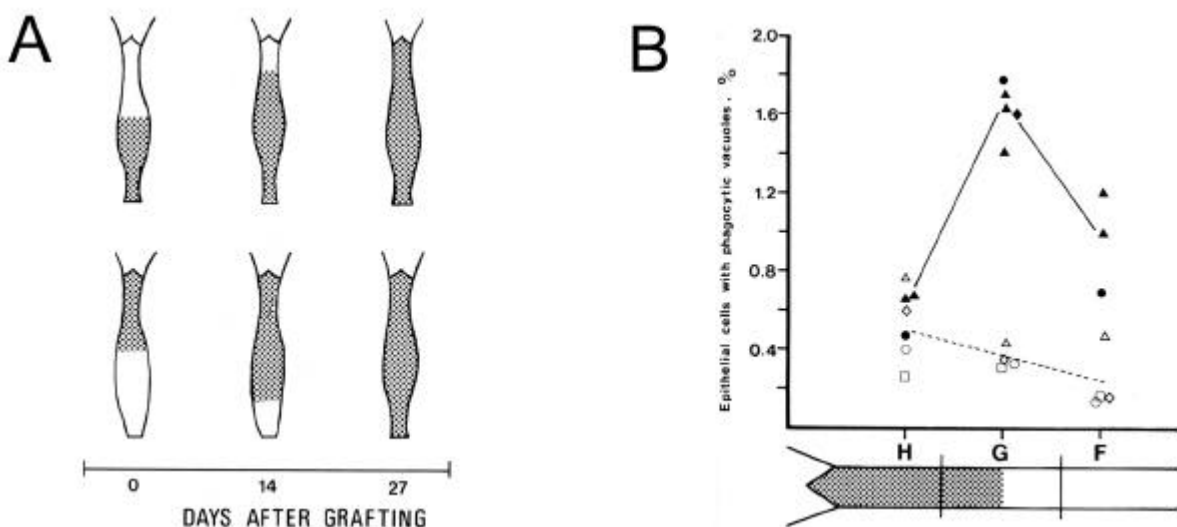


Figure 1.12. Epithelial cells recognize and eliminate non-self by phagocytosis. (A) *H. oligactis* tissue (stippled) displaces tissues of *H. vulgaris* (clear) in interspecific grafts. **(B)** Number of active phagocytes is increased in the graft region in interspecific grafts (solid line) when compared to intraspecific grafts (dotted line) (From Bosch and David, 1986).

Epithelial cells were suggested to perform the recognition function and eliminate foreign cells by phagocytosis (Bosch and David, 1986) Increased number of

active phagocytes was found where cells of the two species confronted one another (Figure 1.12. B). As apoptosis is frequently followed by phagocytosis as described in chapter 1.1.3, I was interested to test and characterize apoptosis in such interspecific grafts.

1.3. Aims of the study

Early steps of evolution of apoptosis are poorly understood. The freshwater cnidarian *Hydra* is the evolutionary oldest animal where the presence of the apoptotic machinery was hitherto documented. However little is known about the processes in *Hydra* where apoptosis is involved. Engagement of apoptosis in regulation of spermatogenesis and self / non-self recognition reactions was anticipated. The aim of my study was to estimate the role of apoptosis and epithelial cells as „non-professional phagocytes“ in these processes in *Hydra*. Specific questions were:

- 1. Does apoptosis take place during spermatogenesis in *Hydra*?**
- 2. Which cells undergo apoptosis in testes?**
- 3. Would the dying cells be eliminated and by which cells?**
- 4. Is apoptosis induced at the graft border in interspecific grafts?**
- 5. What could be the cause of apoptosis induction?**
- 6. Do allogeneic tissues induce apoptosis / phagocytosis?**

2. Material

2.1. Animals

Hydra oligactis, strain Munich

Hydra vulgaris, strain Basel

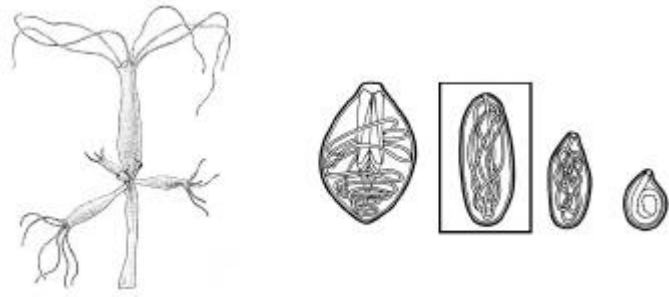
Hydra vulgaris, strain PA2

Hydra vulgaris, strain Ploen

Diagnostic features for *H. oligactis* and *H. vulgaris*:

***H. oligactis* Pallas**

- 1) yellow-orange pigmentation in the endoderm
- 2) stalk-like peduncle
- 3) the tubule of the capsule of holotrichous isorhiza nematocytes is coiled longitudinally



***H. vulgaris* Pallas**

- 1) pink-orange pigmentation in the endoderm
- 2) peduncle is not stalk-like
- 3) the first 3-6 turns of the tubule of the capsule of holotrichous isorhiza nematocytes are coiled transversely

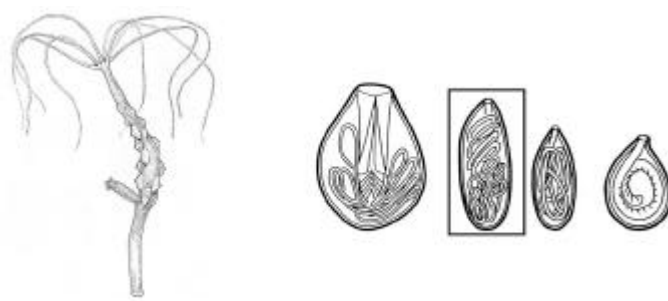


Figure 2.1 Modified from Holstein, 1995

2.2. Media

Artemia medium	31,8 g Seasalt / 1 l ddH ₂ O
Dissociation medium (DM-medium)	3.6 mM KCl, 6 mM CaCl ₂ , 1.2 mM MgSO ₄ , 6 mM sodium citrate, 6 mM sodium pyruvate, 6 mM glucose, 12.5 mM TES, 50 mg/l Rifampicin, pH 6.9
Hydramedium	1 mM CaCl ₂ , 1 mM NaCl , 0.1 mM MgSO ₄ , 0.1 mM KCl , 1 mM Tris-HCl, pH 7.8
LB medium+ampicillin	20 g LB Broth Base , add. 1 l ddH ₂ O , autoclave , 1 ml Ampicillin (50 mg/ml) [For plates add 15 g Bactoagar before autoclaving, pour 10 – 15 ml / plate]
SOB-Medium	20 g Bacto-Trypton, 5 g Yeast extract , 0.58 g NaCl , 0.19 g KCl in 1 l ddH ₂ O
SOC-Medium	10 ml SOB-Medium , 100 µl 2 M Glucose (sterile) , 100 µl MgCl ₂ , 10 mM MgSO ₄

2.3. Buffers and Solutions

Alkaline phosphatase buffer	100 mM Tris (pH 9.5), 50 mM MgCl ₂ , 100 mM NaCl, 0.1% Tween 20, 5 mM Levamisol
-----------------------------	--

Ampicillin-stock	50 mg/ml
IPTG Stock solution	200 mg/ml
Lead citrate	Dissolve 1.33 g $\text{Pb}(\text{NO}_3)_2$, 1.76 g $\text{Na}_3(\text{C}_6\text{H}_5\text{O}_7)\cdot 2\text{H}_2\text{O}$ in ddH ₂ O, mix 30 minutes, add 8 ml 1M NaOH and fill up to 50 ml with ddH ₂ O.
Maceration solution	acetic acid : glycerol : water (1 :1 :13)
PBS : Glycerol	PBS : Glycerol (1 :1)
PBS/Triton/BSA	2 mg/ml BSA, 0.1% Triton X-100 in PBS
PBS : Tween	0.1% Tween-20 in PBS
PCR Puffer (10x)	Amersham Pharmacia Biotech
TAE (50x)	2 M Tris-Acetate, 50 mM EDTA, pH 8.0
TBE (2.5x)	225 mM Tris (27.5 g/l), 225 mM boric acid (13.75 g/l), 5 mM EDTA (10 ml 0.5 M EDTA (pH 8.0) / l)
TE	10mM Tris/HCl pH 7.5, 1mM EDTA pH 8.0
X-Gal	20 mg/ml in DMFA

2.4. Enzymes

- EXCEL II DNA Polymerase Epicentre Technologies
- Proteinase K Sigma
- T4 DNA-Ligase (+ PEG) Promega
- Taq DNA-Polymerase Amersham Pharmacia
Biotech
- Taq DNA-Polymerase Gibco BRL
- Terminal Deoxynucleotidyl Transferase (TdT) Amersham Pharmacia
Biotech

2.5. Kits

- Agar 100 resin Agar Scientific
- High Pure Plasmid Isolation Kit Roche
- Historesin Embedding Kit Leica Instruments
- SequiTherm EXCEL II DNA Sequencing Kit-LC Epicentre Technologies

2.6. Chemicals

- Acridine orange
- Agarose Sigma
- Ampicillin Merck
- APS Roth
- Azur II Merck
- Bactoagar Roth
- Benzol Merck
- Borax Merck
- Boric acid Roth
- Bromphenolblue Fluka
- BSA Merck
- Canada balsam Merck

• Chloroform	Roth
• DEPC (Diethylpyrocarbonate)	Roth
• DMFA	Roth
• DNA Loading buffer (6x)	Fermentas
• DNA-Molecular size marker (100 bp ladder)	Fermentas
• dNTP	Fermentas
• Eosin	Merck
• Ethanol	Roth
• Ethidiumbromide	Merck
• Euparal	Merck
• Formaldehyde (stabilized 37% solution)	Roth
• Gelatine	Sigma
• Glycerol	Roth
• Glytaraldehyde	Serva
• HCl	Merck
• IPTG	Sigma
• LB Broth Base	Life Technology
• Lead acetate	Merck
• Light green	Merck
• Magnesium chloride, 50 mM	Gibco
• Methylbezoate	Merck
• Methylene blue	Merck
• NBT/BCIP concentrate	Boehringer Mannheim
• Osmium tetroxide	Plana W. Plannet GmbH
• Paraffin (Paraplast-plus)	Eydam
• Pioloform	Plana
• Potassium pyrosulfite	Merck
• Propylenoxide	Plana
• Schiff's reagent	Sigma
• Sequagel complete buffer reagent	National Diagnostics
• Sequagel XR	National Diagnostics
• Sodium cacodylate	Fluka
• Sodium citrate	Merck

• TEMED	Merck
• Tris base	Roth
• Triton X-100	Merck
• Tween-20	Merck
• Uranylacetate	Plana
• Urethane	Sigma
• X-Gal	Sigma
• Xylencyanol	Fluka
• Xylol	Merck

2.7. Immunochemicals

Anti-digoxigenin Fab-fragment, coupled to AP	Boehringer Mannheim
Anti- <i>H. vulgaris</i> polyclonal antiserum	A gift from M. Samoilovich
Anti- <i>H. oligactis</i> polyclonal antiserum	A gift from M. Samoilovich
Goat-anti-mouse, coupled with 10 nm gold particles	Sigma
Sheep-anti-mouse, FITC labelled	Boehringer Mannheim

2.8. Vectorplasmids

• pGEM-T	Promega
----------	---------

2.9. Bacteria

E.coli XL-1 Blue

2.10. Oligonukleotides

• Sp6	5'-ATT TAG GTG ACA CTA TAG AAT AC 3'
• T7	5'-TAA TAC GAC TCA CTA TAG GG 3'
• Cnox-2_vul_F	5'-GAACCAGGTGAAGGTACCTCTTC-3'
• Cnox-2_vul_R	5'-CTGAATGCTAGTATACGCGGTTTCG-3'
• OPA 1	5'-CAGGCCCTTG
• OPA 2	5'-TGCCGAGCTG

- OPA 3 5'-AGTCAGCCAC
- OPA 4 5'-AATCGGGCTG
- OPA 5 5'-AGGGGTCTTG
- OPA 6 5'-GGTCCCTGAC
- OPA 7 5'-GAAACGGGTG
- OPA 8 5'-GTGACGTAGG
- OPA 9 5'-GGGTAACGCC
- OPA 10 5'-GTGATCGCAG

All the oligonucleotides were ordered in MWG Biotech

2.11. Electrophoresis

- Agarose-Electrophoresis chamber Omni Separation Systems

2.12. Thermocyclers

- Cyclone Gradient Peq-Lab
- Primus 96 *plus* MWG Biotech
- Primus 25 MWG Biotech

1.13. Centrifuges

- Biofuge 13 Heraeus Instruments
- Eppendorf 5415 D Eppendorf
- Eppendorf 5417 R (cooling centrifuge) Eppendorf
- Minifuge RF Heraeus Sepatech

2.14. Incubators

- Thermoincubator Heraeus Instruments
- Thermoshaker Certomat H B. Braun

2.15. UV-devices

- IMAGO compact imaging system B & L Systems

- UV-Contactlamp Chroma 43 Vetter GmbH
- UV-Stratalinker 1800 Stratagene

2.16. Devices for histology and microscopy

- Electron microscope CM10 Philips
- Electron microscope EM 208 S Philips
- Digital camera AxioCam Zeiss
- Fluorescence microscope Axioskop 2 Zeiss
- Microtome Jung AG (Heidelberg)
- Ultratome Ultracut E Leica

2.17. Miscellaneous

- Artemia salina cysts
- Bio Photometer Eppendorf
- Chelex-100 resin Sigma
- Chromatographic papier Whatman
- Diamond knife C.Schmied-Labortechnik
- Electroporation cuvetts Bio-Rad
- Eppendorf tubes Sarstedt
- Fishing line public shops
- Fluoresbrite plain microspheres,
1 µm diameter, FITC labelled Polysciences
- GenePulser Bio-Rad
- Glass slides Menzel-Gläser
- Grids for electron microscopy Plana
- LI-COR Gene ReadIR 4200 MWG Biotech
- nail-like polypropylene microcentrifuge tubes Roth
- pH-Meter pH 211 Hanna Instruments
- Plastic dishes for hydra culturing Geida
- Plastic sieves (mesh size 0.5 mm) public shops
- Polyethylene tubing
(I.D. 0.38 mm; O.D. 1,09 mm) Becton Dickinson

- Pulselectroporator BioRad
- Sequencing machine
- Rubber form for embedding, Flacheinbetung Plana
- Spectrophotometer Mod. 100-40 Hitachi
- Vortex Genie 2 Scientific Industries

3. Methods

3.1. Handling with animals

3.1.1. Culture condition

Animals were cultured in hydramedium at 18°C with 12 hour light cycle according to the standard method (Lenhoff and Brown, 1970). Each *Hydra* strain was kept in a separate 20 x 20 cm large plastic dish. Polyps were fed 5 days a week with newly hatched and rinsed with freshwater nauplia of brine shrimps *Artemia salina*. Artemia larvae were hatched from cysts during 24-48 hours of incubation in artemia medium at 32°C. Food rests were washed away and dishes were cleaned 6 hours after feeding. All animals were last fed 24 hour prior to be used in experiments.

3.1.2. Grafting procedure

Grafts were produced as described (Rubin and Bode, 1982). Animals to be grafted were cut transversely in the mid-gastric region, and complementary apical and basal halves of two polyps were threaded on a fishing line held together by sleeves of polyethylene tubing to facilitate healing. After 1-2 hours grafts could be taken off the fishing line and maintained under standard conditions without feeding.

3.1.3. Preparation of heteroaggregates

Polyps were mechanically dissociated into a cell suspension in dissociation medium and reaggregated essentially as described (Gierer et al., 1972). To prepare heteroaggregates about 250 polyps of both *Hydra vulgaris* and *Hydra oligactis* were separately dissociated in 20 ml DM-medium. For this animals were gently rubbed with a finger through a plastic sieve with 0.5 mm mesh size under DM-medium. Tissue pieces and large cell clusters were allowed to sink for 30 seconds, and nail-like polypropylene microcentrifuge tubes (Roth) were filled with 400 µl of the remaining cell suspension and centrifuged at 1200 g for 5 minutes at 18°C. The size of resulting pellets allowed us to assess and equilibrate cell concentrations in both preparations. For heteroaggregates equal volumes of *Hydra vulgaris* and *Hydra oligactis* cell suspensions were carefully mixed together, filled in microcentrifuge

tubes and pelleted as described above. The DM-medium was then diluted with hydramedium to 75%, 50%, 33%, 25%, 10%, 5% in 6, 8, 20, 24, 32, and 36 hours after reaggregation, respectively. 44 hours after reaggregation aggregates were transferred into the pure hydra medium. Homoaggregates were always prepared in each experiment to control the size and the quality of the preparation.

3.1.4. Preparation of macerates

Maceration of *Hydra* tissues enabled analysis of distinct cell types. Maceration preparations were performed according to David (1972). Polyps were transferred into a 1.5 ml Eppendorf tube, excessive hydramedium was removed and approximately 20 µl of maceration solution per animal was added. The tubes were incubated at 32°C for 15 minutes with occasional gentle shaking. In the course of this incubation tissues disintegrated to single cells. The cells were then fixed with equal volume of 8% formaldehyde. The resultant cell suspension was distributed over a square space on a glass slide pre-treated with 1% gelatine solution. To reduce surface tension of the cell suspension a small drop of 1% Tween-20 detergent was placed on a glass slide prior to the cell suspension. After the preparations dried they could be stained by histological methods or analysed with phase contrast microscopy without additional.

3.1.5. Tissue labelling with fluorescent beads

Fluorescent latex beads labelled with FITC were used to fluorescently label hydra cells by phagocytosis as described previously (Technau and Holstein, 1992). Endodermal epithelial cells could be selectively labelled by injecting a 2.5% bead suspension into the gastric cavity using a glass capillary. For selective labelling of ectodermal epithelial cells 24-hours starved *H. vulgaris* polyps were incubated for 15 hours in a 0.025% beads suspension. Surprisingly, ectodermal cells of *H. oligactis* could not be labelled by the same procedure. Prior to use all experimental animals were carefully washed with hydramedium.

3.2. Histological methods

3.2.1. Embedding in paraffin and sectioning

At a certain time after reaggregation heteroaggregates were relaxed in urethane for one minute and then fixed with 4% formaldehyde in hydramedium. Dehydration was carried out by incubating samples for one hour in each 70%, 80%, 90%, 100%, 100% EtOH. At the last step a few crystals of eosin have been added to ethanol solution to facilitate tissue recognition during embedding. Samples were then incubated in three changes of methylbenzoate for two hours each. After incubation in two changes of benzol, each for 10 min, samples were transferred into a 1:1 mixture of benzol and paraffin (Paraplast-plus, Eydram) for 10 min at 30°C. Thereafter samples were infiltrated with paraffin by incubating 3 x 2 hours at 56°C. Preparations were hardened in ice-cold water, blocks were trimmed and set up on a wooden form ready to be sectioned. 5-7 µm thick sections were prepared with a conventional microtome (Jung AG, Heidelberg) and spread on a drop of clean water on a glass slide (Menzel-Gläser), which was pre-treated with egg albumin. Sections were stuck to the glass slide when water dried out.

3.2.2. Embedding in historesin

„Historesin Embedding Kit“ was used as an embedding resin. The advantage of the resin over paraffin is that embedded tissue is clearly visible and histological stainings can be carried out directly on a section surface without the need to remove an embedding medium. Resin was prepared according to the manufacturers protocol. 50 ml Basic Resin Liquid was mixed with 0.5 g Activator and stirred overnight at 4°C. Animals were fixed as described above. Dehydration was carried out by incubating animals 2 x 20 min in each 30%, 50%, 70%, 80%, 90% and 96% EtOH. Tissues were infiltrated for 1.5 hours in a mixture of 2 parts 96% EtOH and 1 part Historesin Solution. Infiltration was then continued for another 1.5 hours in 1:1 mixture of historesin and 96% EtOH and then additional 1.5 hours in a 2:1 mixture. Then animals were transferred into a pure historesin solution and incubated overnight. Polymerisation solution was prepared by mixing 3 ml Historesin and 0.2 ml hardener and stirred for 5 minutes. Animals were incubated for 1 hour in a new portion of Historesin and then transferred into polymerisation solution poured in rubber forms.

After incubating 1 day at 4°C followed by 1 day at 60°C the resin was polymerised. 2-5 µm sections were prepared as described above.

3.2.3. Electron microscopy

3.2.3.1. Embedding

One-day-old *Hydra vulgaris* - *Hydra oligactis* heterografts were relaxed in 2% urethane for a few minutes, then fixed with 3.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, for 18 hours at 4°C. After washing with 0.075 M cacodylate buffer for 30 minutes animals were postfixed with 1% OsO₄ in 0.075 M cacodylate buffer for 2 hours at 4°C. After additional washing for 30 minutes animals were dehydrated. For that they were consecutively incubated for 15 minutes in each 50%, 60%, 70%, 80%, 90%, 100%, 100% EtOH. After incubating 2 x 30 minutes in 1,2-propylenoxide it was proceeded with infiltration with Agar 100 resin (Agar Scientific, Ltd., Essex), which is an equivalent of Epon 12. Components of the embedding resin were mixed according to the manufacturers protocol for the "hard" version. Animals were infiltrated for 30 minutes in 1:1 mixture of propylenoxide and Agar 100 resin followed by additional incubation for 30 minutes in 1:3 mixture at room temperature. Incubation in the pure Agar 100 resin was carried out overnight at 4°C. Finally samples were placed into rubber forms in a new portion of the resin and polymerised at 60°.

3.2.3.2. Sectioning: semi-thin sections

Blocks with embedded tissues were trimmed with a razor blade and, first, semithin sections were cut at the Ultracut E ultratom (Leica) to roughly evaluate tissue morphology. Semithin sections were cut with a glass knife and captured on a glass slide. They were stained according to Richardson et al. (1960) with a solution containing 0.5% methylene blue, 0.5% borax, and 0.5% Azur II in ddH₂O. Staining was carried out at 60°C for 1-2 minutes. Here basophilic and osmiophilic structures were stained blue while metachromatic substances were red-violet.

3.2.3.3. Sectioning: ultrathin sections

Ultrathin sections were prepared with the same ultratom with a diamond knife. Sections were captured on round grids previously treated with 1.2% pioloform dissolved in chloroform.

3.2.3.4. Contrasting

To enhance the contrast of fine structures seen under electron microscope, ultrathin sections were additionally stained with uranium and lead. For that ultrathin sections were first treated with 2.5% uranylacetate solution for 5 minutes, rinsed with ddH₂O, and then treated with lead citrate solution (prepared freshly from lead acetate and sodium citrate) for 2 minutes (Reynolds, 1963).

3.2.3.5. Microscopy

Ultrathin sections were analysed under a transmission electron microscope CM10 or EM 208 S (Philips).

3.3. Staining procedures

3.3.1. Histochemical procedures

3.3.1.1. Methylene blue / basic fuchsin

5-7 µm thick sections of testes tissue embedded into Histo-resin were directly stained without removal of the resin. Thus, staining occurred only on the surface of a section. First, sections were stained for 1 hour on a heating plate in a solution containing 2% methylene blue dissolved in 1% aqueous borax. After sections were washed with ddH₂O they were counterstained with 1% aqueous solution of basic fuchsin. Methylene blue intensively stained nuclei while fuchsin stained cytoplasm.

3.3.3.2. Feulgen staining

Feulgen staining enables quantitative staining of DNA in the cells. The principle of the reaction is that under weak acidic condition DNA becomes depurinated thus making polynucleotide aldehyde groups accessible for further reaction with fuchsinsulfuric acid (Schiff's reagent). The resultant product has an intensive red-violet coloration.

Glass slides with maceration preparations (macerates) were washed in ddH₂O for 10 minutes and then acidic hydrolysis was carried out by incubating in 1N HCl at 60°C for 12 minutes. After rinsing in cold ddH₂O nuclei were stained for 1 hour in the Schiff's reagent (Sigma). The preparations were then washed twice in SO₂ containing solution (200 ml tap water, 10 ml 1N HCl, 10 ml 10% potassium pyrosulfite K₂S₂O₅). After additional washing in ddH₂O for 10-15 minutes cytoplasm was stained in 1% light green (Merck) for 2 minutes and preparations were dehydrated in alcohol series: 15 sec in 50% EtOH, 30 sec in 90% EtOH, 2 x 2 min in 100% EtOH. To remove alcohol rests, preparations were incubated 2 x 2 min in xylol and then embedded in Canada balsam (Merck).

3.3.2. Immunohistochemical procedures

3.3.2.1. Removal of cross-species reacting antibodies

Species-specific antibodies (mouse polyclonal antisera) raised against membrane fractions of *H. vulgaris* or *H. oligactis* had approximately 40-50% crossreactivity (Samoilovich et al., 2001). To remove cross-species reacting antibodies from the anti - *H. vulgaris* – antiserum it was preincubated with fixed *H. oligactis* material. For this 20 *H. oligactis* polyps were dissociated into single cell suspension in 1 ml DM-medium as described above and fixed by adding 100 µl of 37% formaldehyde. In 2 hours fixed cells were washed 5 times with PBS-Tween by repeated centrifugation for 3 min at 5000 g and resuspension. After the last centrifugation the pellet was resuspended in 0.5 ml PBS-Tween, 3-5 µl anti – *H. vulgaris* were added and

incubated overnight at 4°C. Before the experiment *H. oligactis* cell material was removed from the antibody solution by centrifuging it twice at 8000 g for 5 minutes. To remove cross-species reacting antibodies from the anti – *H. oligactis* antiserum it was preincubated the same way with fixed cells of *H. vulgaris*.

3.3.2.2. Immunostaining on paraffin sections

To rehydrate paraffin embedded material, glass slides with sections were incubated twice in xylol for 10 minutes. Then they were transferred into 100% EtOH for 15 minutes followed by 5 minutes incubation first in 90% EtOH and then in ddH₂O. Unspecific protein sticking was blocked by incubating the sections in PBS-Tween containing 1% BSA. All incubations were carried out in a humid chamber. Preparations were then covered with 150 µl antiserum solution and incubated overnight at 4°C. The next day preparations were washed from excessive antibodies by incubating in four changes of PBS-Tween for 1 hour each at room temperature. To visualize the primary antibodies, preparations were covered with FITC-labelled secondary sheep-anti-mouse antibodies (1:50 dilution; Boehringer Mannheim Biochemica) and incubated overnight at 4°C. The next day they were washed as previously and counterstained by incubating for 30 minutes in a solution of toluidine blue (several crystals per ml). Tissues stained with toluidine blue had a red fluorescence when viewed with FITC-filter set. Thus, tissues specifically stained with FITC-labelled antibodies appeared green while unlabelled tissues appeared red due to the background staining with toluidine blue. Stained preparations were embedded in 1:1 solution of PBS:Glycerol and viewed under fluorescence microscope with FITC filter set.

3.3.2.3. Immunostaining on ultrathin sections

Cross-species reacting antibodies were removed from a specific antiserum as described above. All incubations were carried out by placing a grid with ultrathin sections on the top of appropriate solution drops put on a piece of parafilm. Prior to contrasting, unspecific binding sites were blocked by incubating the grids in PBS

containing 0.1% Triton and 3% BSA. (PBS-Triton-BSA) for 30 minutes. Incubation with the primary antibody diluted as above in PBS-Triton-BSA was carried out at 37°C for 1 hour. Preparations were then washed 4 x 5 minutes with PBS-Triton-BSA and incubated with the secondary goat-anti-mouse antibodies (Sigma) coupled with 10 nm large gold particles (1:20 dilution in PBS-Triton-BSA) for 40 minutes at 37°C. Preparations were then washed 4 x 5 minutes in PBS-Triton-BSA followed by washing 4 x 1 minute with ddH₂O. Then the preparations were contrasted with uranylacetate and lead citrate as described.

3.3.2.4. Whole mount staining with antibodies

One-day-old heterografts were relaxed for 1 minute in 2% urethane and fixed with 4% formaldehyde for at least 4 hours at 4°C. Then they were washed with PBS. The following staining procedure was exactly as it was described for paraffin sections, except for that the counterstaining with toluidine blue was not necessary. Stained animals were mounted on glass slides in 1:1 solution of PBS:Glycerol and viewed under fluorescence microscope with FITC filter set.

3.4. Apoptosis assays

3.4.1. Acridine orange staining

Acridine orange is a cationic acidophilic dye, which specifically stains apoptotic cells within phagocytic vacuoles with low pH value (Schwarz and Wittekind, 1982; Clerc and Barenholz, 1998). Living *Hydra* polyps were incubated in 0.1µM solution of acridine orange in hydramedium for 15 minutes. Then the animals were washed with hydramedium 4 times and mounted on a glass slide in a drop of 2% urethane solution. Living animals were then viewed under fluorescence microscope with a FITC filter set.

3.4.2. TUNEL staining

DNA fragmentation is a hallmark of apoptosis and can be revealed by biochemically labelling free DNA termini by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling) method (Gavrieli *et al.*, 1992).

Hydra polyps were relaxed in 2% urethane and fixed with 4% formaldehyde for 2 hours at room temperature. After initial washing in PBS for 5 minutes animals were permeabilized by incubating 2 x 15 minutes in PBS containing 1% Triton X-100. The solution was then replaced by PBS-Tween and animals were incubated 2 x 15 minutes. Additional incubation with PBS was carried out twice for 15 minutes. Before the end-labelling reaction, animals were incubated for 1 hr in 200 μ l 1 x TdT buffer (Roche) at room temperature. The solution was replaced with 100 μ l TdT buffer containing 0.5 μ M digoxigenin-labelled dUTP and 150 U/ml TdT-enzyme. The reaction was allowed to continue for 2 hours at 37°C and then stopped by incubating 2 x 1 hour in PBS/EDTA solution, at 65°C. Animals were then washed 4 x 1 hour with PBS at room temperature and then in PBS/Triton/BSA for 15 min. To block unspecific antibody binding sites, animals were incubated in the blocking buffer (25% inactivated sheep serum in PBS-Tween) for 1 hour at room temperature. The solution was then replaced with the blocking buffer containing a 1:5000 dilution of anti-digoxigenin antibody coupled to alkaline phosphatase. Incubation was carried out overnight at 4°C. The next day animals were washed 6 x 1 hour with PBS-Triton-BSA. Then animals were incubated 2 x 5 minutes in the alkaline phosphatase buffer and transferred into a well of a 24 well plate. Solution was replaced with 1 ml alkaline phosphatase buffer containing NBT/BCIP diluted 1:50. After the colour reaction developed, it was stopped by replacing the solution with 96% EtOH. Finally the animals were embedded in Euparal (Merck) and analysed under the microscope. TUNEL staining on macerates was carried out according to the same procedure.

3.5. Molecular techniques

To estimate precisely the degree of phylogenetic divergence between the three strains of *H. vulgaris*, molecular techniques were applied.

3.5.1. Extraction of genomic DNA for PCR

Genomic DNA was extracted from one tentacle of a single polyp using chelating resin Chelex-100 (Walsh et al., 1991). This method of genomic DNA isolation was preferred over conventional ones because it was simple and enabled analysis of individual variability between the animals.

One tentacle of each polyp was cut and transferred into a 200 μ l PCR-tube. All hydramedium was removed and 25 μ l of 10 mM Tris-HCl buffer, pH 11, containing 20% (w/v) Chelex-100 resin (Sigma) were added. The mixture was boiled for two hours in a PCR-machine with a hot-top (Primus 96 *plus*, MWG Biotech). During this time the tube was occasionally shaken to facilitate tissue rupture.

The resultant crude extract of genomic DNA could be directly used as a template for a PCR reaction. Alternatively, it could be stored shortly at 4°C or longer at -20°C.

3.5.2. PCR-based methods

3.5.2.1. RAPD analysis

RAPD (Rapid Amplification of Polymorphic DNA) analysis represents a low-stringency PCR-based method to randomly amplify a number of genomic fragments in a single reaction. Subsequent analysis of the pattern of amplified products allows to quantitatively estimate differences between samples (Williams et al., 1990).

I used RAPD method to estimate gross genomic differences between polyps of the three different strains of *H. vulgaris*. Crude extracts of genomic DNA were prepared from at least 5 polyps of each strain as described above, and used as a template for PCR amplification. PCR reaction was prepared as delineated in the table below:

<i>Component</i>	<i>Volume (μl)</i>	<i>Final concentration</i>
10 x PCR Buffer (Amersham)	1	1 x
Template DNA	0.5	----
Primer (10 μ M)*	0.2	0.2 μ M
MgCl ₂ (50 mM)	0.1	2 mM **
dNTPs (1 mM each)	1	100 μ M
Taq Polymerase (5U/ μ l)(Amersham)	0.1	0.05 U/ μ l
ddH ₂ O	7.1	-----
Total	10	

(*) – decamer primers with a randomly chosen sequence were used (OPA-1 through OPA-10)

(**) – The 1 x PCR Buffer (Amersham) contained 1.5 mM MgCl₂

Amplification was carried out in a „Primus 96 plus“ (MWG Biotech) PCR-machine with the maximum ramping time according to the following programme:

- 1) Denaturation 3 min 95°C
- 2) 45 cycles:
 - Denaturation 40 sec 95°C
 - Annealing 1 min 36°C
 - Elongation 1 min 72°C
- 3) Elongation 2 min 72°C

After the reaction was completed, 2 µl 6 x DNA loading buffer was added and amplification products were separated in 1% agarose gel containing 0.03% ethidium bromide and viewed under UV-illumination.

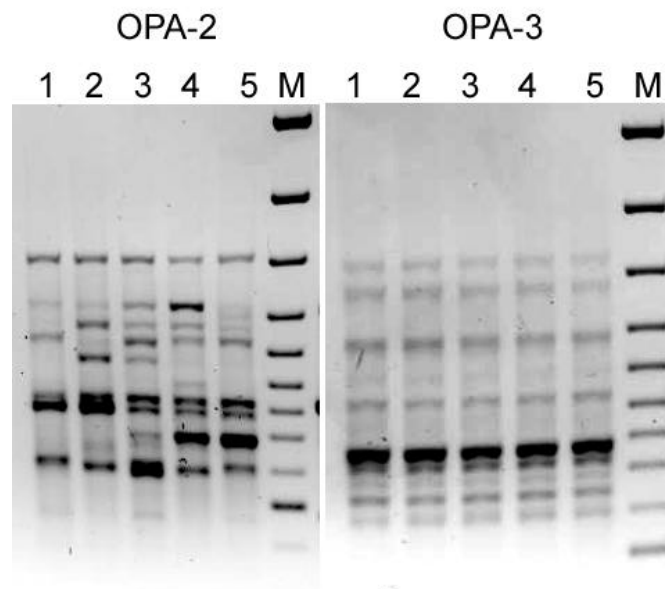


Fig. 3.1 Variable and stable RAPD patterns from 5 individual *H. vulgaris* Ploen polyps with two different primers. 1-5 - different individuals. OPA-2 and OPA-3 – primers used for amplification.

Several primers have been tested in RAPD analysis. Some of them such as OPA-2 revealed individual differences between polyyps within one strain, while others such as OPA-3 produced a stable pattern for each strain (Figure 3.1).

3.5.2.2. Amplification with sequence-specific primers

As an alternative to the RAPD method, quantitative estimation of genomic differences between the *H. vulgaris* strains could be achieved by direct sequencing of a comparable genomic segment from all three strains. An intron recently recognized in the Cnox-2 gene of *H. vulgaris* was chosen as a reference. As an intron and thus a non-coding region, it might contain nucleotide substitutions even in very close populations. Two sequence-specific primers were designed based on the published sequence of the Cnox-2 homeobox gene from *H. vulgaris* (GenBank accession number AJ277388):

forward primer Cnox-2_vul_F 5'-GAACCAGGTGAAGGTACCTCTTC-3'
reverse primer Cnox-2_vul_R 5'-CTGAATGCTAGTATACGCGGTTTCG-3'

<i>Component</i>	<i>Volume (µl)</i>	<i>Final concentration</i>
10 x PCR Buffer (Gibco)	2	1 x
Template DNA	1	----
Forward primer (10 µM)	2	1 µM
Reverse primer (10 µM)	2	1 µM
MgCl ₂ (50 mM)	0.8	2 mM
dNTPs (25 mM)	0.2	250 µM
Taq Polymerase (5U/µl) (Gibco)	0.2	0.05 U/µl
ddH ₂ O	12	-----
Total	20	

Amplification was carried out in the Primus 96 *plus* (MWG Biotech) thermocycler according to the following programme:

- | | |
|-----------------|-------------|
| 1) Denaturation | 2 min 95°C |
| 2) 35 cycles | |
| Denaturation | 45 sec 95°C |
| Annealing | 45 sec 63°C |
| Elongation | 45 sec 72°C |
| 3) Elongation | 2 min 72°C |

After the reaction was completed 10 µl of the resultant mixture were mixed with 2 µl DNA loading buffer and analysed in an agarose gel. If a single clear band of approximately expected size was seen, an aliquot from the remaining PCR reaction mixture was used to directly ligate the fragment into pGEM-T vector.

3.5.3. Agarose gel-electrophoresis

PCR fragments, depending on the expected size, were separated in agarose gels containing between 1% and 1.5% agarose. To visualize DNA-bands ethidiumbromide was included into the gels (0.03%). 1 x TAE was used as a running buffer. After DNA-loading buffer was added to the samples, DNA fragments were separated electrophoretically usually at 70-90 V. The size of DNA fragments was estimated under UV-illumination according to the comigrating DNA-size standards such as 100 base pair ladder.

3.5.4. Cloning

3.5.4.1. Ligation into pGEM-T vector

T/A-cloning is based on the ability of a Taq-polymerase to add an additional adenine to the 3'-end of a double-stranded DNA fragment. PCR fragments with A-overhangs can be easily cloned into linearized vectors with T-overhangs at the 5'-ends, such as pGEM-T.

Ligation into pGEM-T vector was carried out according to the manufacturers (Promega) protocol. 3 µl PCR product were added to 5 µl Ligation Buffer, 1 µl vector, and 1 µl T4-Ligase. Reaction was carried out at 4°C for more than 16 hours.

3.5.4.2. Competent cells

Electrocompetent cells of *E. coli* XL-1 Blue strain were used for transformation. To prepare them, a single colony of this strain was grown overnight at 37°C in 3 ml SOB-Medium containing 12 µg/ml tetracyclin. 2 ml of the overnight culture were transferred into 50 ml SOB-Medium and incubated at 37°C for further 3-4 hours until they reach logarithmic phase ($OD_{600}=0.5-0.6$). After centrifugation 10 minutes at 3000 g bacterial pellet was washed with 50 ml cold ddH₂O. Then bacteria were centrifuged once more and washed first with 25 ml cold ddH₂O and then with 2 ml of cold 10% glycerol. After the last pelleting (10 minutes, 3000g) bacteria were resuspended in 200 µl 10% glycerol, divided into 40 µl-aliquots and frozen at -80°C.

3.5.4.3. Transformation

For electroporation of bacteria, electroporation cuvettes (Bio-Rad, distance between electrodes 1 mm) were taken out of the storage solution (70% EtOH), washed several times with ddH₂O and irradiated in UV-crosslinker to destroy the remaining DNA. The cuvettes were cooled on ice before usage and SOC-Medium was warmed to 37°C. 40 µl – aliquots of electrocompetent cells were thawed on ice. 1 µl ligation product was added to the cells, mixed thoroughly and immediately transferred into a cooled electroporation cuvette. The electroporation parameters were set at 1.7 kV, 25 µF and 200 Ohm. Optimal impulse time reached 4.0 – 5.0 msec. After electric pulsing bacteria were immediately mixed with 1 ml warm SOC-Medium and shaken at 37°C for one hour. 200 µl of the cell suspension were then plated out onto LB-Ampicillin plates and incubated overnight at 37°C. For white / blue selection of bacterial clones containing vector with an insert, 50 µl X-Gal (50 mg/ml) and 100 µl IPTG (100 mM) were plated on a plate before transformation.

3.5.4.4. Insert-check

To prove whether white colonies contained the correct insert, the length of the fragment was checked by PCR reaction with vector-specific primers (Sp6 and T7). A single overnight grown colony was picked from an agar-plate and transferred into a 200 μ l –PCR tube containing the following components:

<i>Component</i>	<i>Volume (μl)</i>	<i>Final concentration</i>
10 x PCR Buffer (Pharmacia)	1	1 x
T7 primer (10 μ M)	0.5	0.5 μ M
Sp6 primer (10 μ M)	0.5	0.5 μ M
MgCl ₂ (50 mM)	0	1.5 mM
dNTPs (10 mM)	0.1	100 μ M
Taq Polymerase (5U/ μ l)	0.1	0.05 U/ μ l
ddH ₂ O	7.8	-----
Total	10	

Amplification was carried out in the Primus 96 *plus* (MWG Biotech) thermocycler according to the following programme:

- 1) Denaturation 2 min 95°C
- 2) 40 cycles
 - Denaturation 45 sec 95°C
 - Annealing 45 sec 52°C
 - Elongation 45 sec 72°C
- 3) Elongation 2 min 72°C

After the reaction was completed, 2 μ l DNA loading buffer were added to the resultant mixture and analysed in an agarose gel as described above.

10 positive clones for each cloned PCR product were then sequenced.

3.5.5. DNA sequencing and sequence analysis

3.5.5.1. Plasmid purification

To isolate plasmids from bacteria the „High Pure Plasmid Isolation Kit“ (Roche) was used. Plasmid purification was carried out from 4 ml overnight bacterial culture according to the manufacturers protocol. First, bacterial cells were lysed in alkaline conditions. At this step bacterial RNA is digested due to the presence of RNase A. In the presence of chaotropic salt guanidiniumhydrochlorid the plasmid-DNA binds specifically to glassfibers on „High Pure Filters“. The bound DNA is cleaned from bacterial substances via repeated quick wash- and centrifugation steps. Finally DNA is eluted from glassfibers with 100 µl low-salt buffer.

3.5.5.2. DNA sequencing

DNA sequencing was carried out using the SequiTherm EXCEL II DNA Sequencing Kit-LC (Epicentre Technologies). This technique is based on the Sanger dideoxymediated chain-termination method (Sanger et al., 1977). Fragments were amplified in a thermocycler with a T7 or Sp6 primer labelled with fluorescent dye IRD-800 at the 5'-end (MWG Biotech) to enable band detection in the automated sequencer. Reactions were set up according to the manufacturers protocol. Sequencing reaction products were separated and detected in a LI-COR Gene ReadIR 4200 (MWG Biotech) automated sequencing machine and analysed with the manufacturers software.

3.5.5.3. Sequence analysis

Sequences of at least 3 clones from each of 3 individuals of each *H. vulgaris* strain were compared with one another and the published sequence of *H. vulgaris* Cnox-2 gene (GenBank accession number AJ277388). Sequences were aligned using ClustalW multiple sequence analysis software, version 1.8 (Thompson et al., 1994) available at the ClustalW internet server of the Baylor College of Medicine under URL: <http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html>. Intron sequence was recognized as a piece absent in the published mRNA sequence. Surprisingly, the second last base before the intron was different in fragments isolated here from that in the published sequence: T(514) and C(514), respectively.

4. Results

4.1. Role of epithelial cells and apoptosis in *Hydra* spermatogenesis

4.1.1. Morphological features of *Hydra* spermatogenesis

Prior to conduct apoptosis assays, testes morphology was re-examined for the *H. vulgaris* PA2 strain. As shown in figure 4.1 A, a male „gonad“ appears as a protrusion in the ectoderm. Covered by the ectodermal epithelial cells, germ cells are organized into layers of differentiating sperm precursors so that precursors of each stage comprise a distinct layer (Figure 4.1 B and C). Spermatogonia comprise the most basal layer of germ line cells adjacent to mesoglea. These are large cells with interphase nuclei which frequently have two nucleoli suggesting high metabolic activity. Primary and smaller secondary spermatocytes are located distally to spermatogonia. These cells undergo meiotic divisions so that condensed chromosomes can be frequently observed in them. Spermatocytes comprise the proximal half of the testis as seen in figure 4.1 B. Distally from spermatocyte layer postmeiotic spermatids begin to differentiate into spermatozoa. Their nuclei assume first the oval shape of the spermatozoan head then the cells form tails thereby becoming mature spermatozoa. Spermatozoa leave testes being ejaculated through a nipple-like structure at the top of the testis.

Ectodermal epithelial cells form not only the envelope of the testis in which spermatogenesis occurs, but are also found between the developing sperm precursors (Figure 4.1 B – D). Epithelial protrusions seem to divide the gonad into peripheral chambers containing mainly spermatogonia and spermatocytes and central chambers mainly occupied by differentiating spermatids and spermatozoa (Figure 4.1 C and D). Due to the long epithelial protrusions, which stretch from the surface towards the mesoglea, interstitial sperm precursor cells appear to be in close contact to epithelial cells all along their differentiation pathway.

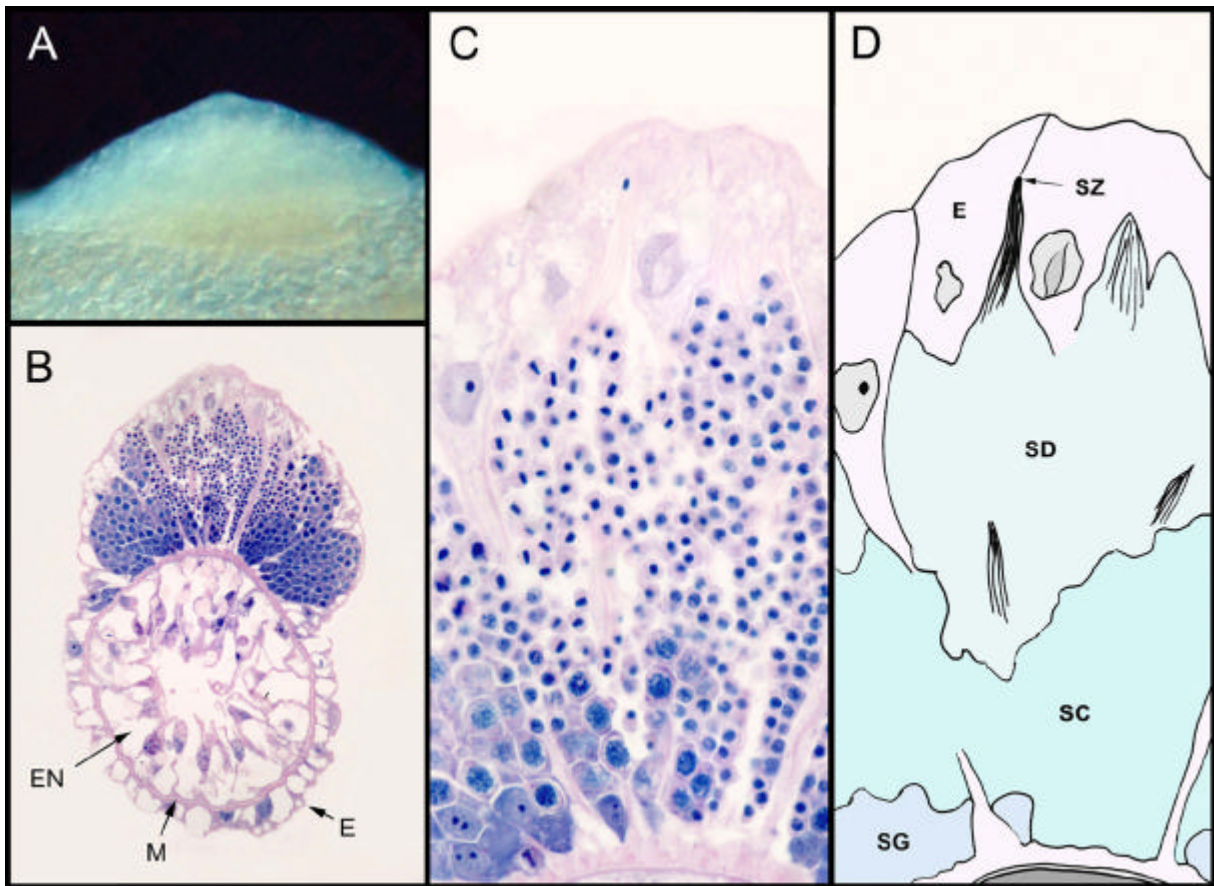


Fig. 4.1 The anatomy of the testis in *Hydra vulgaris*. (A) Testis viewed under phase-contrast illumination; (B) Methylene blue / basic fuchsin / borax stained section of mature testes. Abbreviations: E - ectoderm; EN - endoderm; M - mesoglea; (C) higher magnification 1000x) of the testis shown in (B). (D) Schematic diagram illustrating the close relationship between epitheliomuscular cells and male germ cells of all developmental stages. Abbreviations: SG - spermatogonia; SC - spermatocytes; SD - spermatids; SZ - spermatozoa; E - ectodermal epitheliomuscular cell.

4.1.2. Apoptosis in *Hydra* testes

In male polyps taken from the stock culture before the onset of sexual differentiation, little or no signs of apoptotic cell death were found in the body column, when stained with the vital dye acridine orange (Figure 4.2 A). In the foot, head and tentacle tissue a slight increase in the number of acridine orange positive cells was observed, which is consistent with previous observations (Cikala et al., 1999) and the fact that hydra cells are dying at the extremities. However in sexually mature male polyps large number of acridine orange positive cells was found within each testis (Figure 4.2 C and E). The cells were not restricted to a certain region of the testis suggesting that cells at various stages of spermatogenesis undergo programmed cell death.

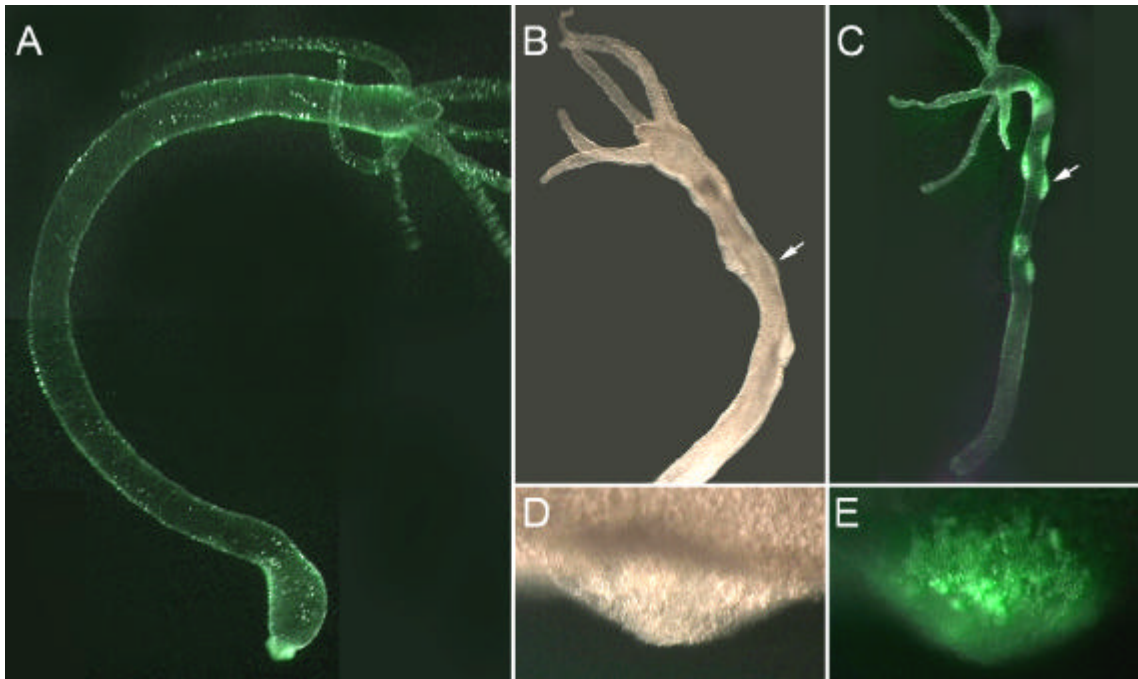


Figure 4.2 Acridine orange specifically stains *Hydra* testes. (A) Control polyp of *Hydra vulgaris*. **(B)** Dark field microscopic view of a sexually mature male *Hydra vulgaris* polyp. **(C)** the same polyp as shown in (B) stained with acridine orange. Signs of massive cell death (green staining) appear in all the testes. Arrow exemplifies one testis. **(D and E)** higher magnification of one of the testes shown in (B and C), respectively. Signs of massive cell death are detectable throughout the whole testes.

Acridine orange is a cationic (acidophilic) dye that stains vesicles with low pH value in living cells (Schwarz and Wittekind, 1982; Clerc and Barenholz, 1998). Thus phagocytosed apoptotic cells are acridine orange positive. However, the opposite is not necessarily true, i.e. we cannot assume that all acridine orange positive cells are apoptotic. Therefore TUNEL assay enabling detection of the nuclei with fragmented DNA, a hallmark of apoptosis, was adopted for *Hydra* (see Materials and Methods). *Hydra* testes stained by TUNEL method are shown in figure 4.3.

Numerous nuclei undergoing DNA fragmentation have been found in *Hydra* testes. In contrast to the acridine orange staining, TUNEL positive cells are located mostly at the periphery of the testes and therefore most likely represent rather early stages of spermatogenesis.

Hydra testes grow in size as the number of sperm precursors within the testis increases. After the mature sperm appears, a nipple is formed by epithelial cells at the

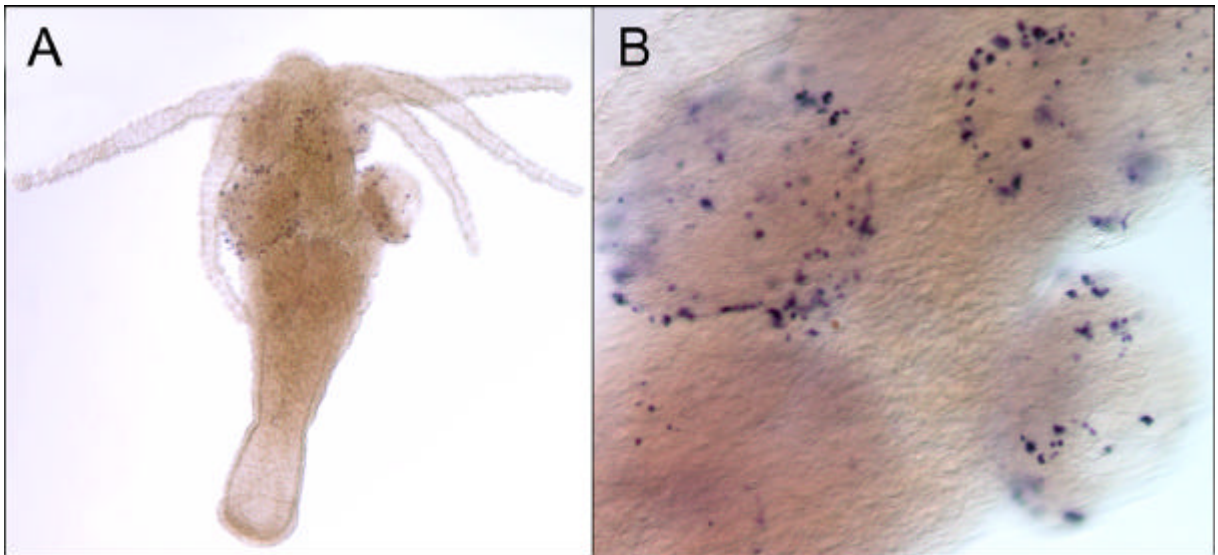


Figure 4.3 Whole-mount detection of DNA fragmentation in male *Hydra vulgaris* by the TUNEL method (A). (B) Higher magnification of the polyp shown in (A) demonstrating numerous TUNEL positive nuclei at the periphery of each testis.

top of the testis allowing the sperm to be ejaculated. Eventually each testis ceases functioning and undergoes involution after 7-8 days (Brien and Reniers-Decoen, 1951). An involuting testis has a narrower basement part than the middle one. Factors regulating growth of one testis and involution of the other simultaneously on the same polyp are unknown. Interestingly, few, if any, TUNEL positive cells have been observed in young testes that did not form a nipple yet (Figure 4.4 A). Conversely, mature testes and testes undergoing involution revealed remarkably many TUNEL positive nuclei, most of which were localized to the proximal layers of the testes (Figure 4.4. B and C).

This suggests that apoptosis is not involved in early steps of spermatogenesis, but is necessary when all types of sperm precursors are well established and later when the whole structure has to be eliminated.

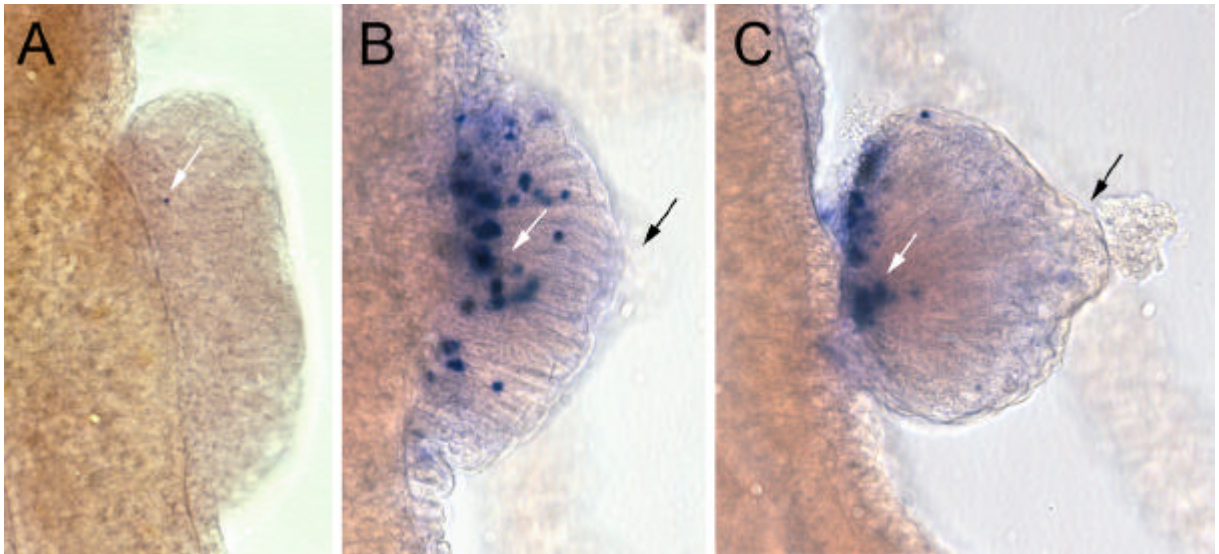


Figure 4.4. Apoptosis at different stages of testes development. (A) Young testis. **(B)** Mature testis. **(C)** Involuting testis. White arrows indicate TUNEL positive cells. Black arrow shows a nipple.

4.1.3. Cell types undergoing programmed cell death

To identify the cell types undergoing apoptosis during spermatogenesis, maceration preparations of sexually mature male polyps were subjected to the TUNEL assay. As shown in figure 4.5, TUNEL positive cells can be found either as single cells (Figure 4.5 D) or in vacuoles within epithelial cells (Figure 4.5 A – C). Single cells comprised 7% of all TUNEL positive cells counted (17 out of 236; Figure 4.5 D). The remaining 93% of the TUNEL positive cells were found to be enclosed in phagocytic vacuoles within epithelial cells with average density of about 2 phagocytosed cells per one epithelial cells. It is more difficult to identify the type of the cells being enclosed in phagocytic vacuole (Figure 4.5 B and C). According to the size and nuclear morphology, however, at least some of the phagocytosed nuclei (54 out of the 219) appear to belong to spermatozoa (Figure 4.5 A). Up to 10 spermatozoa could found enclosed in one epithelial cell. The observation, that spermatozoa are phagocytosed during spermatogenesis was confirmed by Feulgen staining (see below, Figure 4.6 D). Thus the remaining 75 % of the TUNEL positive cells must represent earlier stages of spermatogenesis.

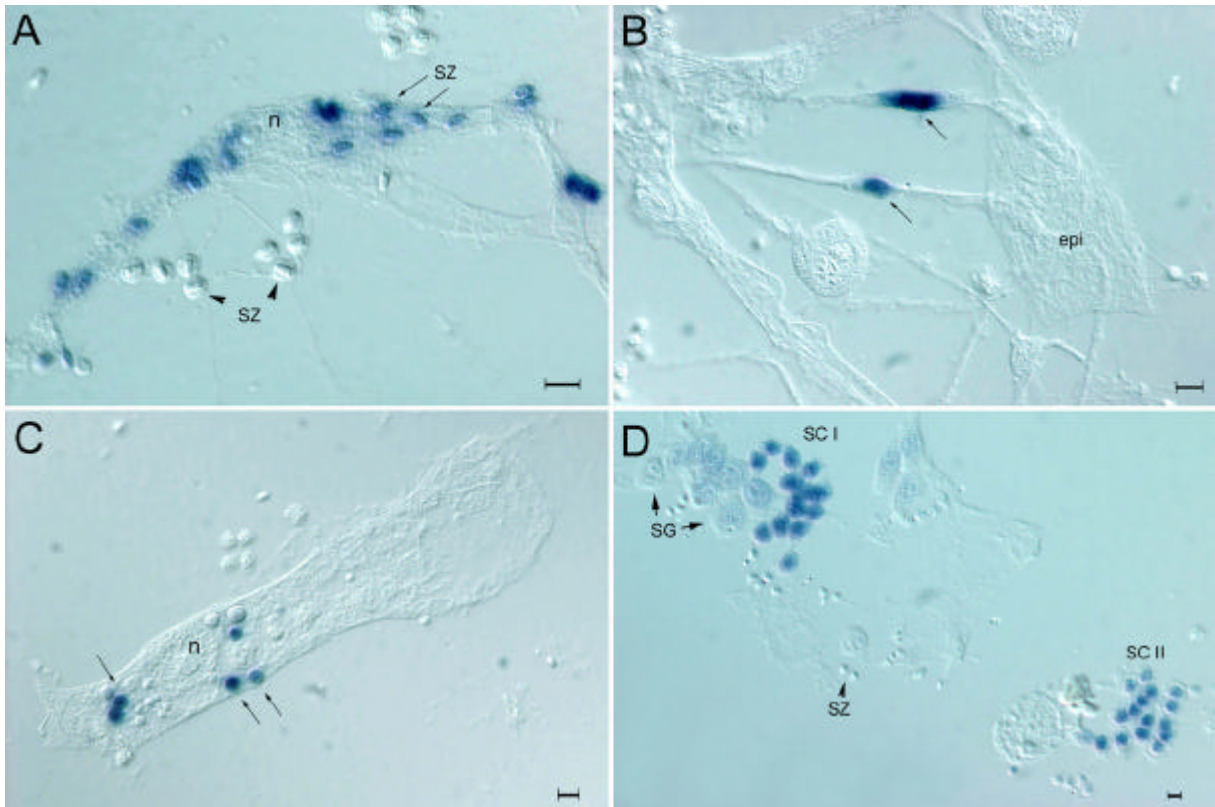


Fig. 4.5 Sperm precursors undergo apoptosis at various stages of spermatogenesis. (A–E) TUNEL stained maceration preparation of sexually mature *Hydra vulgaris* male. **(A)** TUNEL positive spermatozoa (arrows) enclosed in phagocytic vacuoles of an epithelial cell. SZ, spermatozoa; n, nucleus of the epithelial cell. Arrowheads indicate TUNEL negative spermatozoa. **(B)** Apoptotic nuclei in long protrusions of ectodermal epithelial cells (epi). **(C)** Epithelial cell containing phagocytosed sperm precursors other than spermatozoa; arrows indicate apoptotic bodies; n, nucleus of the epithelial cell. **(D)** Apoptotic spermatocytes prior to engulfment. SG, spermatogonia; SC I, primary spermatocytes; SC II, secondary spermatocytes; SZ, spermatozoa.. Scale bar corresponds to 2 μ m.

4.1.4. Epithelial cells act as phagocytes and remove apoptotic sperm precursors

Identification of apoptotic sperm precursor cells revealed that these cells are engulfed and eliminated by ectodermal epithelial cells. Figure 4.5 demonstrates TUNEL positive nuclei within phagocytic vacuoles of epithelial cells. While most of the engulfed nuclei are located in the central part of the epithelial cell close to the epithelial nucleus (Figure 4.5 A and C), TUNEL stained nuclei occasionally could also be found in the long epithelial protrusions (Figure 4.5 B). This is surprising as the long protrusions of epithelial cells were thought to form just physically supporting pillars for a protruding testes (see figure 4.1 B and C).

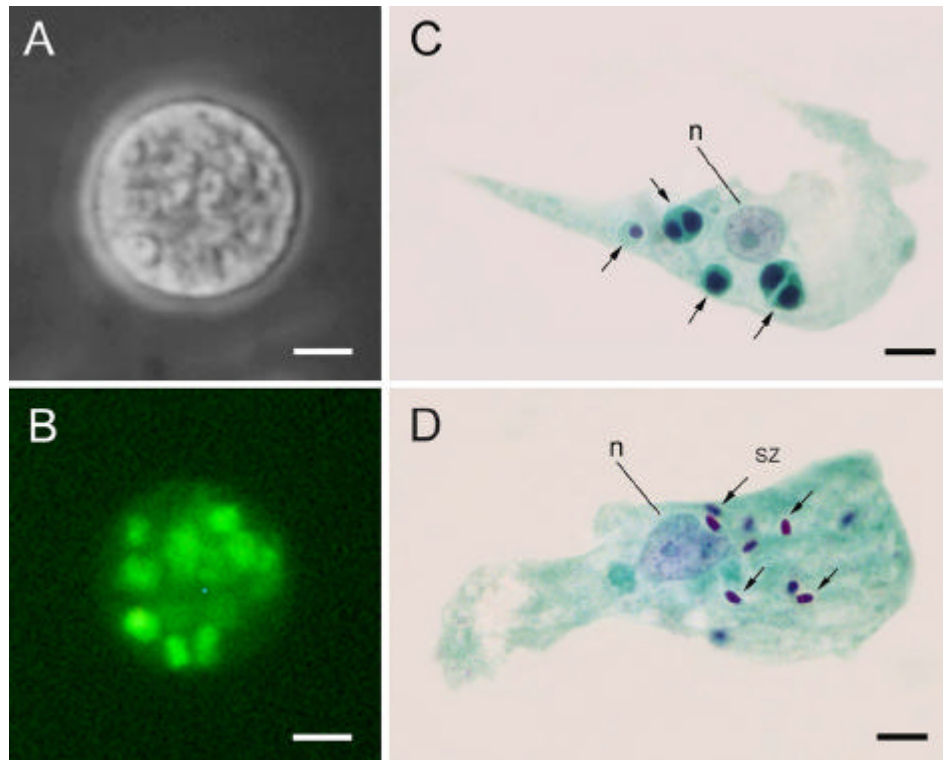


Fig. 4.6. Ectodermal epitheliomuscular cells function as phagocytes and eliminate apoptotic sperm precursor cells. (A) Epithelial cell after pronase treatment viewed under phase contrast; **(B)** The cell shown in (A) stained with acridine orange. **(C)** Feulgen stained epithelial cell containing several apoptotic bodies (arrows); n, nucleus of the epithelial cell. **(D)** Feulgen stained epithelial cell containing spermatozoa (arrows); n, nucleus of the epithelial cell. Scale bar corresponds to 3 μm .

To support the apoptosis data obtained by the TUNEL assay, male polyps were also examined by staining cell preparations with acridine orange and Feulgen method. When testes were dissociated by pronase treatment and stained with acridine orange, about 5% of the ectodermal epitheliomuscular cells contained large numbers of acridine positive vacuoles (Figure 4.6 A and B). In some cases the form and size of the acridine orange positive material enclosed into such vacuoles was somewhat reminiscent of those of spermatozoan heads (Figure 4.6 B). Analysis of Feulgen stained preparations (Figure 4.6 C – D) provides additional evidence that *Hydra* ectodermal epitheliomuscular cells serve as phagocytes to eliminate apoptotic sperm precursors.

4.2. Role of apoptosis in interspecific grafts in Hydra

4.2.1. Apoptosis is induced in interspecific grafts

Increased incidence of phagocytosis in the graft area was reported previously for *H. vulgaris* – *H. oligactis* heterografts and proposed to be a mechanism for epithelial cells to eliminate non-self cells (Bosch and David, 1986). In order to examine whether phagocytosis is preceded by induction of apoptosis in such heterografts, grafts between *H. oligactis* and *H. vulgaris* Basel strain were prepared, and subjected to apoptosis detection assays 24 hours after grafting.

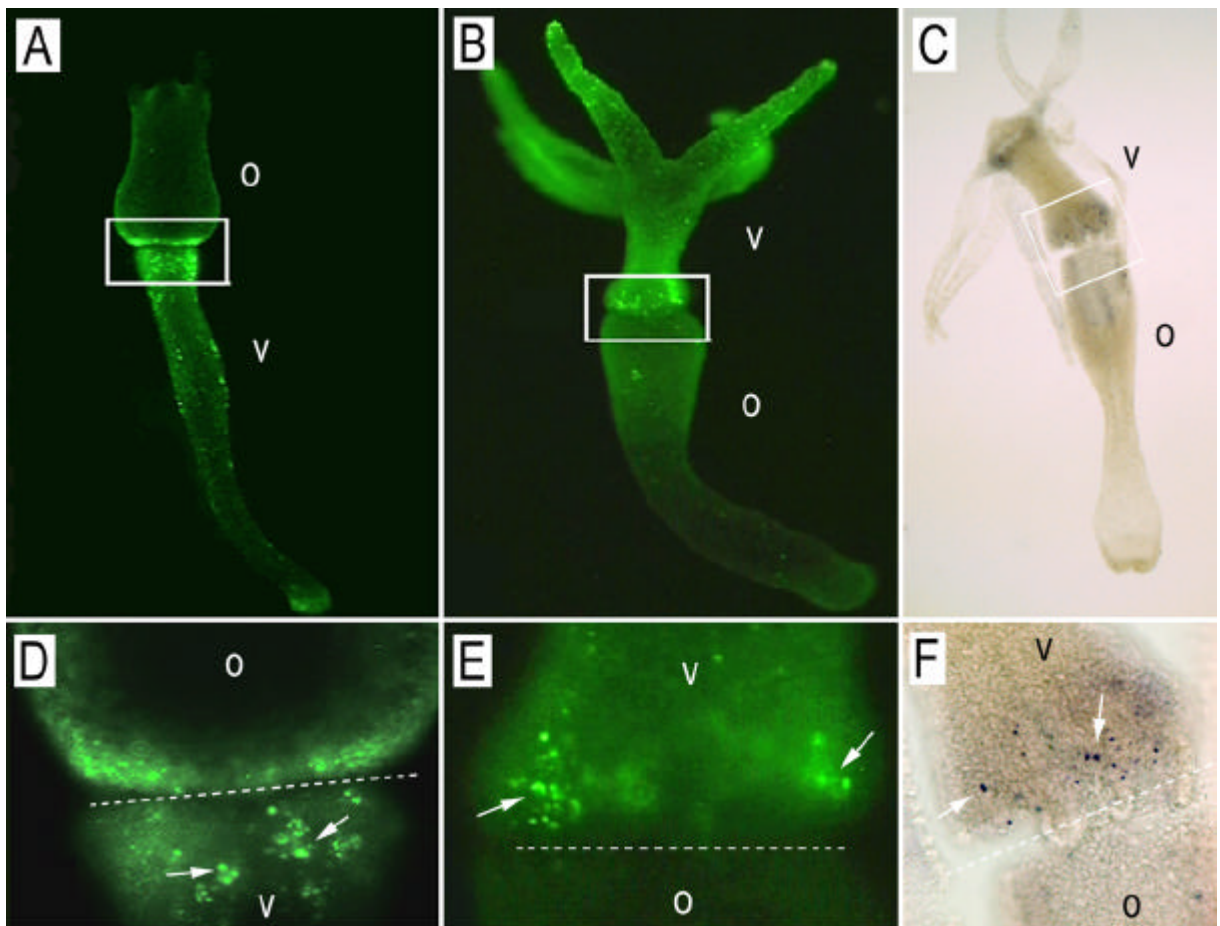


Fig. 4.7 Apoptosis is induced at the graft border in heterografts. (A and B) – 24-hours-old heterografts vitally stained with acridine orange. **(C)** - a 24-hours-old heterograft stained by TUNEL method. **(D – F)** graft sites indicated by frames in (A – C), respectively, under higher magnification. (o) – *H. oligactis* tissue; (v) – *H. vulgaris* tissue. Dotted line indicates the graft border. Arrows show acridine orange- or TUNEL-positive cells undergoing apoptosis. Note that most of them are apparently restricted to the *H. vulgaris* tissue adjacent to the graft border.

Rare apoptotic cells were found scattered throughout the entire body of polyps in heterografts and homografts, and some more of them were usually detected in tentacles and in the foot region, where cell deaths normally occur (Figure 4.7 A, B and Figure 4.8). Interestingly, *H. oligactis* polyps appeared to contain generally fewer apoptotic cells when compared to *H. vulgaris*, suggesting that cell susceptibility to death signals may be different in these two species.

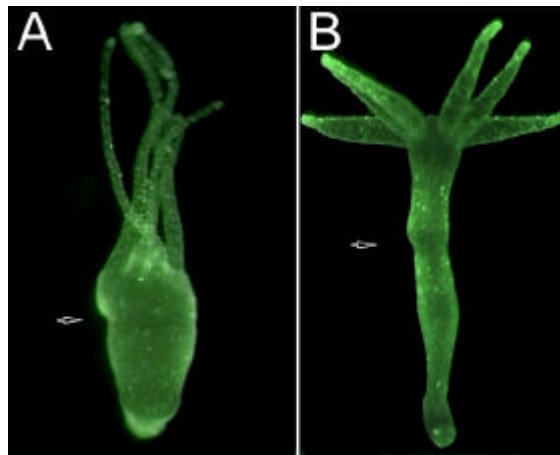


Fig. 4.8 Apoptosis is not induced in control homografts. 24-hours-old homografts of *H. oligactis* – *H. oligactis* (A) and *H. vulgaris* – *H. vulgaris* (B) vitally stained with acridine orange. Arrows indicate the grafting site recognizable as a constriction line in the mid-gastric region.

Both vital staining with acridine orange and TUNEL assay revealed increased number of apoptotic cells at the graft site in heterografts when compared to control homografts (Figure 4.7 and 4.8). In heterografts apoptotic cells were found restricted to the *H. vulgaris* portion adjacent to the graft border irrespective of the species orientation in the grafts, i.e. whether one species was placed distally or proximally relative to the other (Figure 4.7 D – F). This indicated that an active process resulting in induction of programmed cell death was initiated when tissues of these two species came in close contact.

In order to get insight into the processes, which cause induction of apoptosis in heterografts, their behaviour and morphology of the grafting site was investigated.

4.2.2. Stability of interspecific grafts

Grafts between different *Hydra* species were long considered as a model for the ancestral form of transplantation immunity (Kolenkine, 1958; Kanaev, 1969). It was shown that depending on species combination grafted pieces either separated after a certain time or stayed indefinitely as a chimera. Though phylogenetically diverged species usually separated when grafted, different outcomes were reported for interspecific grafts between *H. vulgaris* and *H. oligactis* (Campbell and Bibb, 1970; Bosch and David, 1986). Therefore stability of heterografts used in this study was assessed.

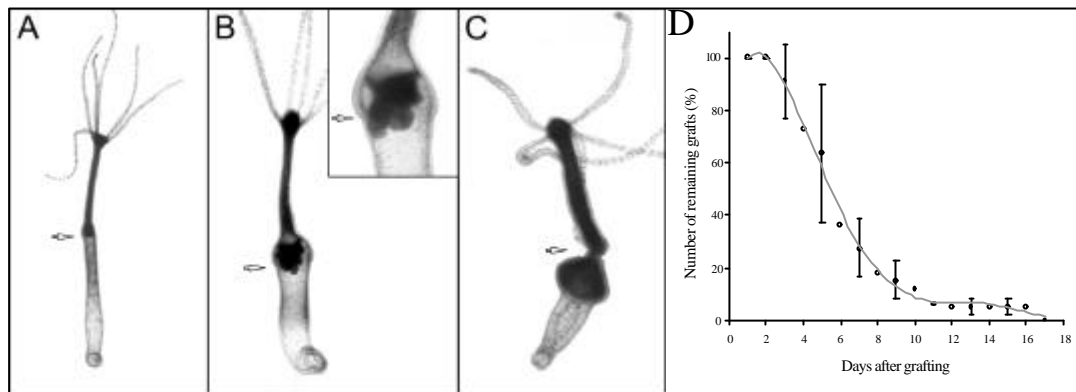


Fig. 4.9 Stability of *H. vulgaris* – *H. oligactis* heterografts after grafting. (A , B and C) – *H. vulgaris* (apical) – *H. oligactis* (basal) heterograft 1 day, 3 days, and 7 days after grafting, respectively. Inlet in (B) is the graft region of the chimera shown in (B) under higher magnification. (D) – Graf showing decrease in stability of heterografts. Values are means \pm S.D. The solid line is the polynomial trend line through the data points.

H. vulgaris – *H. oligactis* heterografts were found to be not permanently stable. Transplanted halves of *H. vulgaris* and *H. oligactis* polyps fused 1-2 hours after grafting and appeared best healed 1 day after the procedure (Figure 4.9 A). However, the graft border could be still identified by a small furrow in the mid-gastric region and a slight constriction. Surprisingly, a clump of tissue became clearly visible in the gastric cavity at the level of the graft border a few days later (Figure 4.9 B). In the course of the next days a constriction advanced at the position of a heterotypic junction in the ectoderm (Figure 4.9 C) eventually resulting in pinching apart of the grafted halves. The dynamics of separation of the heterografts is shown in figure 4.9 D. All of the 179 *H. vulgaris* – *H. oligactis* grafts in 5 independent experiments eventually fell apart within the period of 3 weeks. The average time of parabiosis was 5.5 days. Control *H. vulgaris* – *H. vulgaris* and *H. oligactis* – *H. oligactis* homografts

never separated. Graft junction perfectly healed a few hours after grafting and was hard to be identified in the next days.

The separation rate between *H. vulgaris* (apical) – *H. oligactis* (basal) heterografts did not differ significantly from the vice versa oriented grafts. Separation dynamics of 84 *H. vulgaris* – *H. oligactis* heterografts and 95 *H. oligactis* – *H. vulgaris* heterografts was compared by pairwise two-side Student's t-test. The probability that they are different had a value of 0.03425, thus below the 5% confidence interval. However, as seen in the graph (Figure 4.9 D), the variability of the values for the period between 2 – 7 days after grafting was relatively high. This seemed to be dependent on the quality of the grafting performance so that the better the grafting was done, the longer both species stayed together.

4.2.3. Morphology of the heterotypic junction in the ectoderm

Richard Campbell proposed (Bibb and Campbell, 1973a), that stability of *Hydra* heterografts was caused by poor adhesion between foreign ectodermal epithelial cells rather than by a dedicated histocompatibility system. In order to test whether poor adhesion undermined graft stability in our case, septate junctions between foreign cells in the ectoderm were investigated at electron microscopic level.

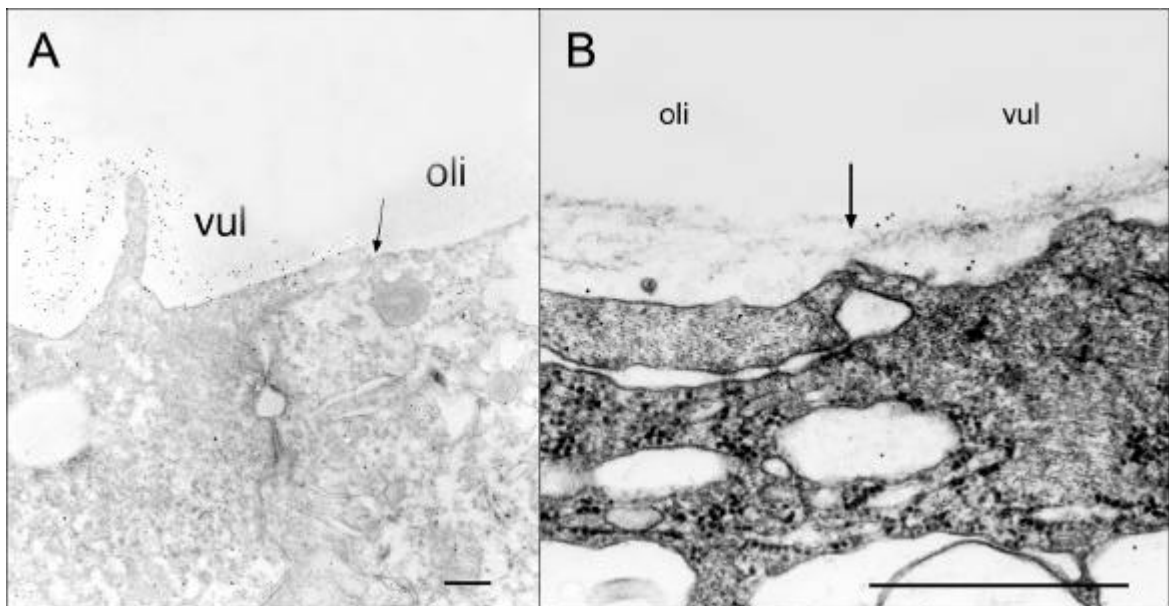


Fig. 4.10 Species-specific antibodies recognize glyocalyx antigens. (A) – Uncontrasted ultrathin section through the *H. vulgaris* – *H. oligactis* graft site. Antibodies coupled with 10 nm large gold particles stain mainly glyocalyx of *H. vulgaris*. **(B)** – The same preparation as in the figure 2.14 stained with gold-coupled

antibodies. Arrows indicate the heterotypic intercellular contact. Scale bar corresponds to 0.5 μm .

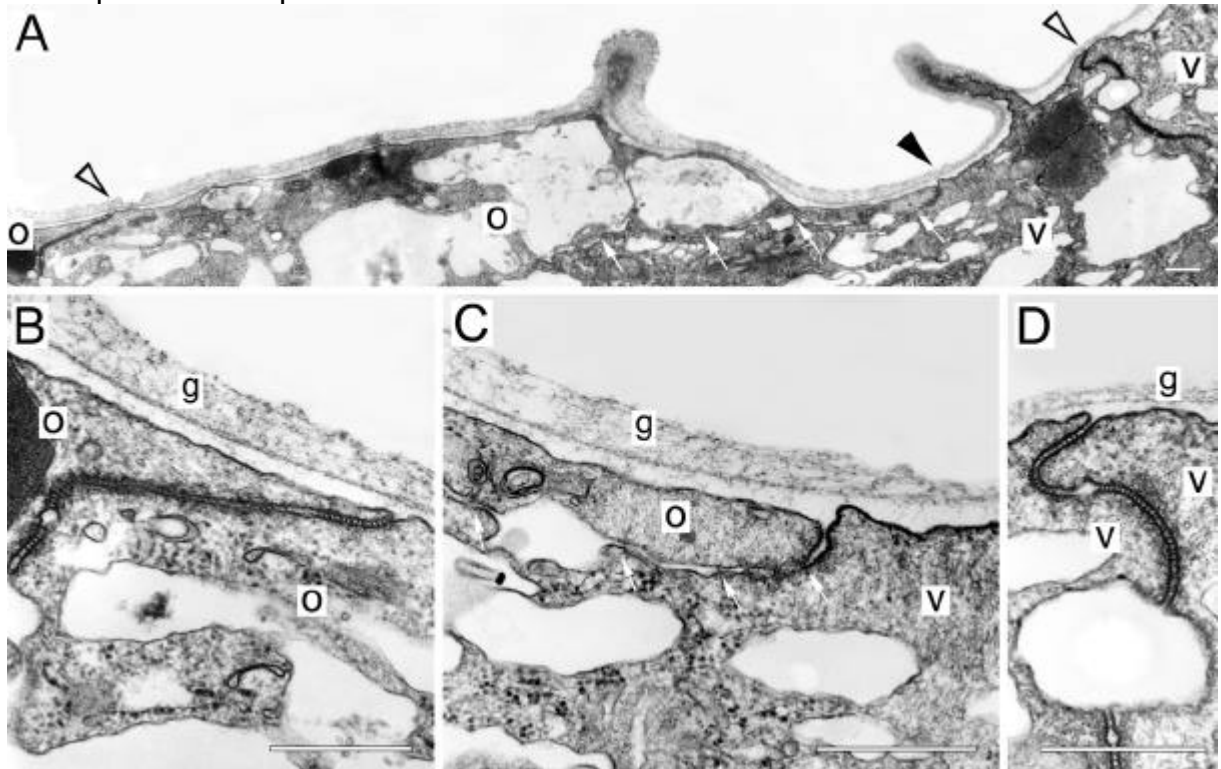


Fig. 4.11 Ultrastructure of homotypic and heterotypic septate junctions in the ectoderm of a 24-hours-old heterograft (A) – **The left and the right white arrow heads show homotypic junctions between two cells of *H. oligactis* (o), enlarged in (B), and two cells of *H. vulgaris* (v), enlarged in (D), respectively. Note regular septae and spaces between cell membranes. (C) – enlargement of the heterotypic junction indicated by the black arrowhead in (A). White arrows in (A) show the course of the heterotypic contact with a few irregular septae and spaces between cell membranes shown in (C). (g) - glycocalyx. Note its species-specific differences. Scale bars correspond to 0.5 μm .**

24-hours-old *H. vulgaris* – *H. oligactis* heterografts were prepared for electron microscopy. To precisely identify the position of the heterologous cell junction, cells of one species were labelled with species-specific antiserum (Samoilovich et al., 2001). This antiserum was raised by immunizing a mouse with membrane fraction of *H. vulgaris*. It proved to strongly label glycocalyx of *H. vulgaris* on ultrathin sections (Figure 4.10). The heterotypic junction could then also be recognized as a cell junction at the transition from one cell with a thick glycocalyx characteristic for *H. oligactis* ectodermal epithelial cells to a cell with a thinner glycocalyx characteristic for *H. vulgaris* ectodermal epithelial cells (Figure 4.10 B and Figure 4.11 A and C). Heterotypic contacts usually appeared as a few scattered septae-like elements in the places of desmosomes. Spaces between cell membranes of the two foreign cells

were highly irregular (Figure 4.11 C). On the contrary, the same cells demonstrated normal morphology of septate desmosomes at the opposite to the heterotypic contact sides where they contacted another cell of the same species (Figure 4.11 B and D). Here the junctions were well extended, with regular septae and spaces between cell membranes as previously described (Wood, 1959).

Thus our data are in line with previous observations on septate junctions in interspecific grafts and suggest that poor adhesion between foreign cells in the endoderm is the true reason of instability of the *H. vulgaris* – *H. oligactis* heterografts.

4.2.4. Differential epithelial movement at the graft site

As mentioned in chapter 4.2.2., a clump of tissue became clearly visible in the gastric cavity at the level of the graft border as soon as 24 hours after grafting and progressed in the course of the next days of parabiosis. No similar observations were found in the literature available to date. We investigated this phenomenon histologically as it might be relevant to induction of apoptosis in this region.

The dark clump could be observed inside at the level slightly shifted into the *H. vulgaris* portion from the grafting site (Figure 4.12 A). The region between the grafting site and the place where the tissue clump apparently touched endoderm of *H. vulgaris* was a little swollen. Longitudinal histological sections through the heterografts revealed that this clump corresponded to the endodermal tissue of *H. vulgaris* detached from the mesoglea and hanging into the gastric cavity (Figure 4.12 B). Thus it seemed that the *H. oligactis* endoderm has moved upwards and displaced the endoderm of *H. vulgaris*.

To test whether *H. oligactis* endoderm in fact moved faster than the ectoderm and partially replaced *H. vulgaris* endoderm in heterografts, the following experiment was performed. First, endoderm of *H. oligactis* polyps was labelled with fluorescent latex beads (see Materials and Methods). Then such *H. oligactis* polyps were grafted with unlabelled *H. vulgaris* polyps in reciprocal combinations. They were fixed 2 or 24 hours after grafting and stained with *H. vulgaris* – specific polyclonal antibodies. These grafts were then examined under fluorescence microscope (Figure 4.13). Here, antibodies clearly stained ectodermal layer of *H. vulgaris*, while fluorescent beads stained endodermal layer of *H. oligactis*. This allowed us to identify borders

between the two species both in ectoderm and in endoderm simultaneously to assess their movements relative to each other.

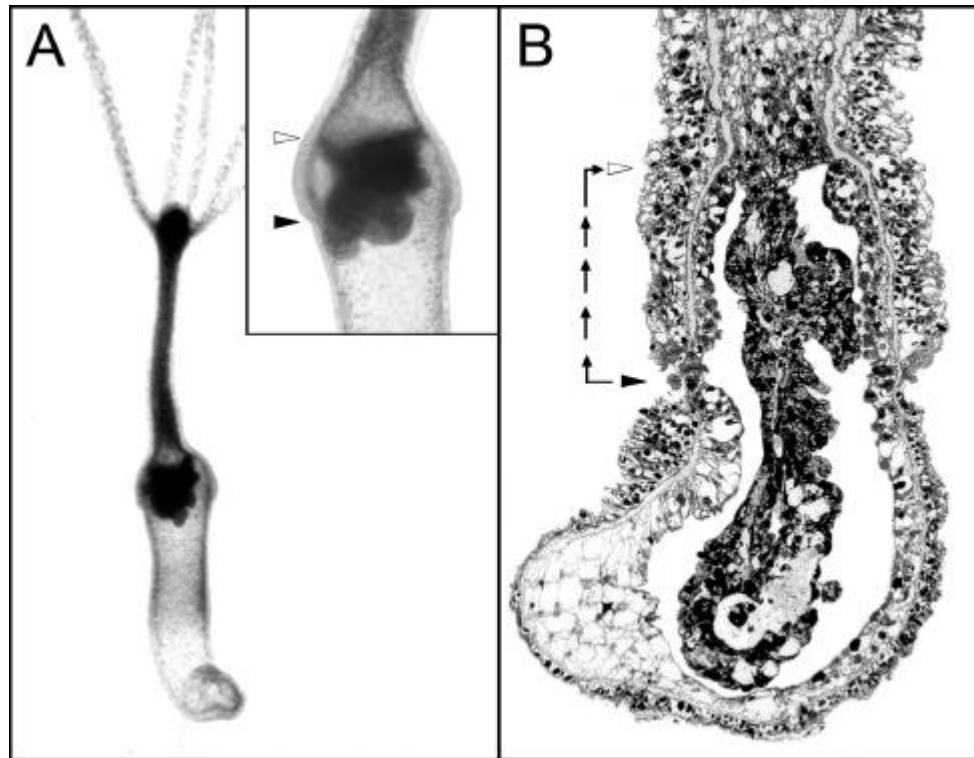


Fig. 4.12 Appearance of the graft site in heterografts. (A) – 1-day-old heterograft with *H. vulgaris* half placed distally and *H. oligactis* proximally. Inlet – the grafting site under higher magnification. **(B)** – longitudinal section through a heterograft similar to that in (A). Note a clump of *H. vulgaris* endodermal tissue hanging into the gastric cavity. Solid arrowhead – heterologous contact in the ectoderm. Open arrowhead – endoderm detachment site. Arrows – movement direction of *H. oligactis* endoderm.

In *H. oligactis* (distal) – *H. vulgaris* (proximal) heterografts contacts between the species both in ectoderm and in endoderm were at the same level 2 hours after grafting (Figure 4.13 B and G). However, 22 hours later a region could be identified where antibody labelled ectoderm (*H. vulgaris*) and fluorescent beads-labelled endoderm (*H. oligactis*) overlapped (Figure 4.13 C and H) suggesting that *H. vulgaris* endoderm was displaced by the endoderm of *H. oligactis* moving downwards. Similarly, in heterografts with the opposite species orientation no overlapping was found early after grafting (Figure 4.13 D and I), while, again, labelled ectoderm partially overlapped beads-labelled endoderm 24 hours after grafting (Figure 4.13 E and J). Here *H. oligactis* endoderm appeared to move upwards. This confirms the observations described previously (Figure 4.12).

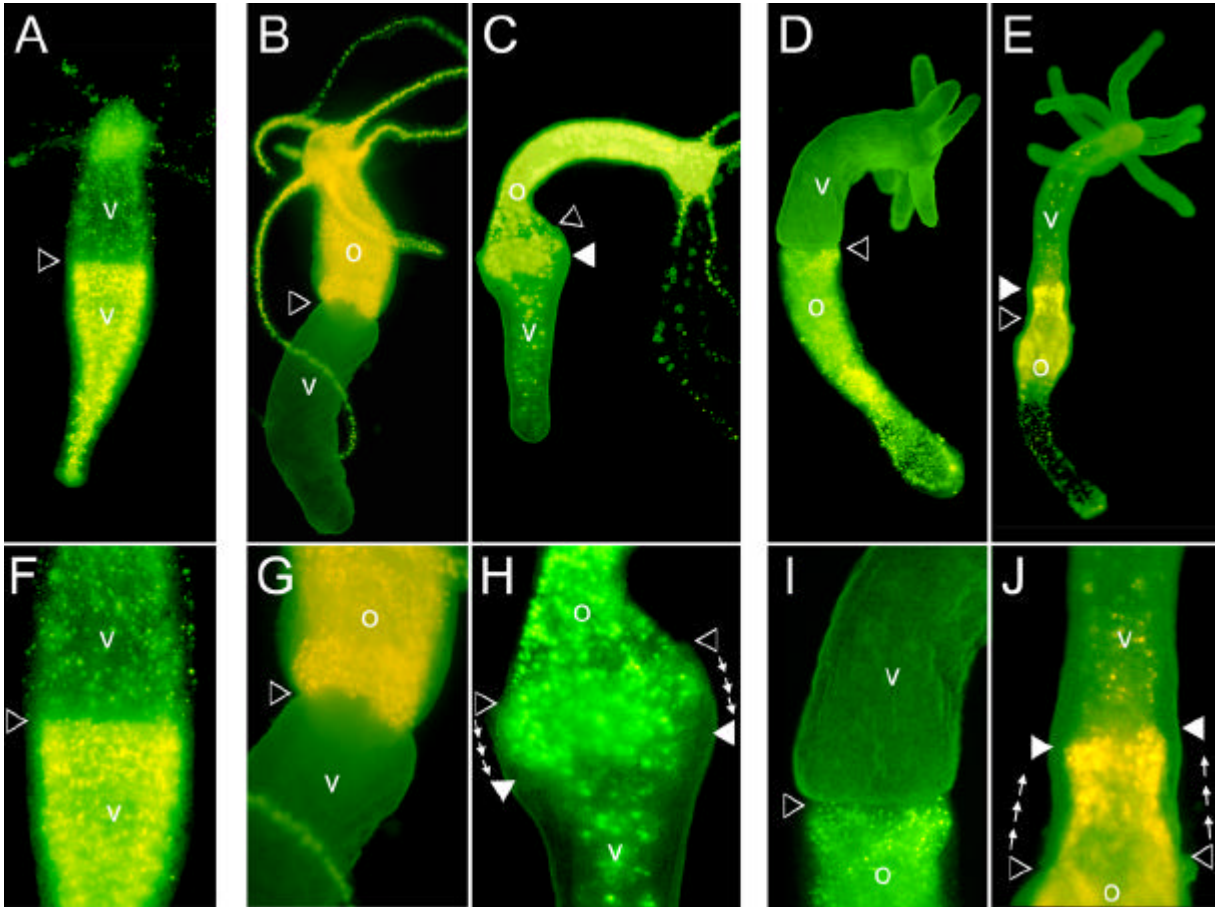


Fig. 4.13 Differential movement of *H. oligactis* endoderm and ectoderm in heterografts. (A and F) - control 24-hours-old *H. vulgaris* homografts. Ectoderm of the upper half and endoderm of the lower half is labelled with fluorescence latex beads. No overlapping or divergence of labelled ectoderm and endoderm = no differential epithelial movement. **(B and C)** *H. oligactis* – *H. vulgaris* heterografts 2 and 24 hours after grafting, respectively. **(D and E)** *H. vulgaris* – *H. oligactis* heterografts 2 and 24 hours after grafting, respectively. **(G – J)** – graft region of the heterografts shown in (B – E) under higher magnification. In (B – E and G – J): *H. oligactis* endoderm is labelled with fluorescence latex beads. *H. vulgaris* ectoderm is labelled with species-specific antibodies. Open arrowheads indicate border between the species in ectoderm. Solid arrowheads – border between the species in endoderm due to differential epithelial movement. Arrows indicate the direction of the movement of *H. oligactis* endoderm.

The extent to which *H. oligactis* endoderm moved beneath *H. vulgaris* ectoderm was different from one individual graft to another. In a few cases *H. oligactis* endoderm reached nearly the hypostome region of *H. vulgaris* half. Nevertheless, usually *H. oligactis* endoderm shifted to only about 1-2 millimetre. The distance for which *H. oligactis* endoderm moved seem to have been correlated positively with the quality of

the grafting performance and was less evident when *H. oligactis* half was grafted apically to *H. vulgaris*.

In control *H. vulgaris* homografts ectoderm of the one half and endoderm of the other half were labelled with fluorescence latex beads. No overlapping or divergence of labelled ectoderm and endoderm was observed 24 hour after grafting, suggesting the absence of significant differential movement of the epithelia (Figure 4.13 A and F). Equivalent control homografts for *H. oligactis* were not investigated because ectodermal cells of *H. oligactis*, surprisingly, did not uptake the latex bead from the medium and, therefore, could not be labelled. However, one may assume that tissue dynamics in *H. oligactis* does not differ too much from that in *H. vulgaris*.

This is the first time when formation of a chimeric region in such heterografts was described, in which ectoderm of one species (*H. vulgaris*) and endoderm of another species (*H. oligactis*) overlapped. As induction of apoptosis in heterografts was restricted to this region, one could speculate that ecto- / endodermal chimerism is involved in this process.

4.2.5. The mesoglea in the chimeric region

Ultrastructural investigation of the chimeric region in 24-hours-old heterografts revealed morphologically abnormal mesoglea separating *H. vulgaris* ectoderm from *H. oligactis* endoderm (Figure 4.14). Under electron microscope the border between ectodermal cells of the two species (heterotypic ectodermal junction) was identified as described above. The border between the species in the endoderm was identified as a place where *H. vulgaris* endoderm detached from the mesoglea (Figure 4.14 A and B). Outside the chimeric region mesogleal layer had relatively even thickness (mo – normal mesoglea in *H. oligactis* in figure 4.14 C; mv – normal mesoglea of *H. vulgaris* in figure 4.14 G). However, in the chimeric region mesoglea appeared highly irregular. It was enormously thick in some places (Figure 4.14 C, D) or even branching (Figure 4.14 F) while in other places it was nearly elusive (Figure 4.14 E) or even broken (Figure 4.14 C).

As mesoglea was shown to play essential role in cell proliferation, cell migration, morphogenesis and cell survival (Sarras et al., 1993; Schmid et al., 1999), its abnormal morphology suggests that some of its functions might be affected. Mesoglea abnormalities in the chimeric region might be the cause of induced apoptosis (see Discussion).

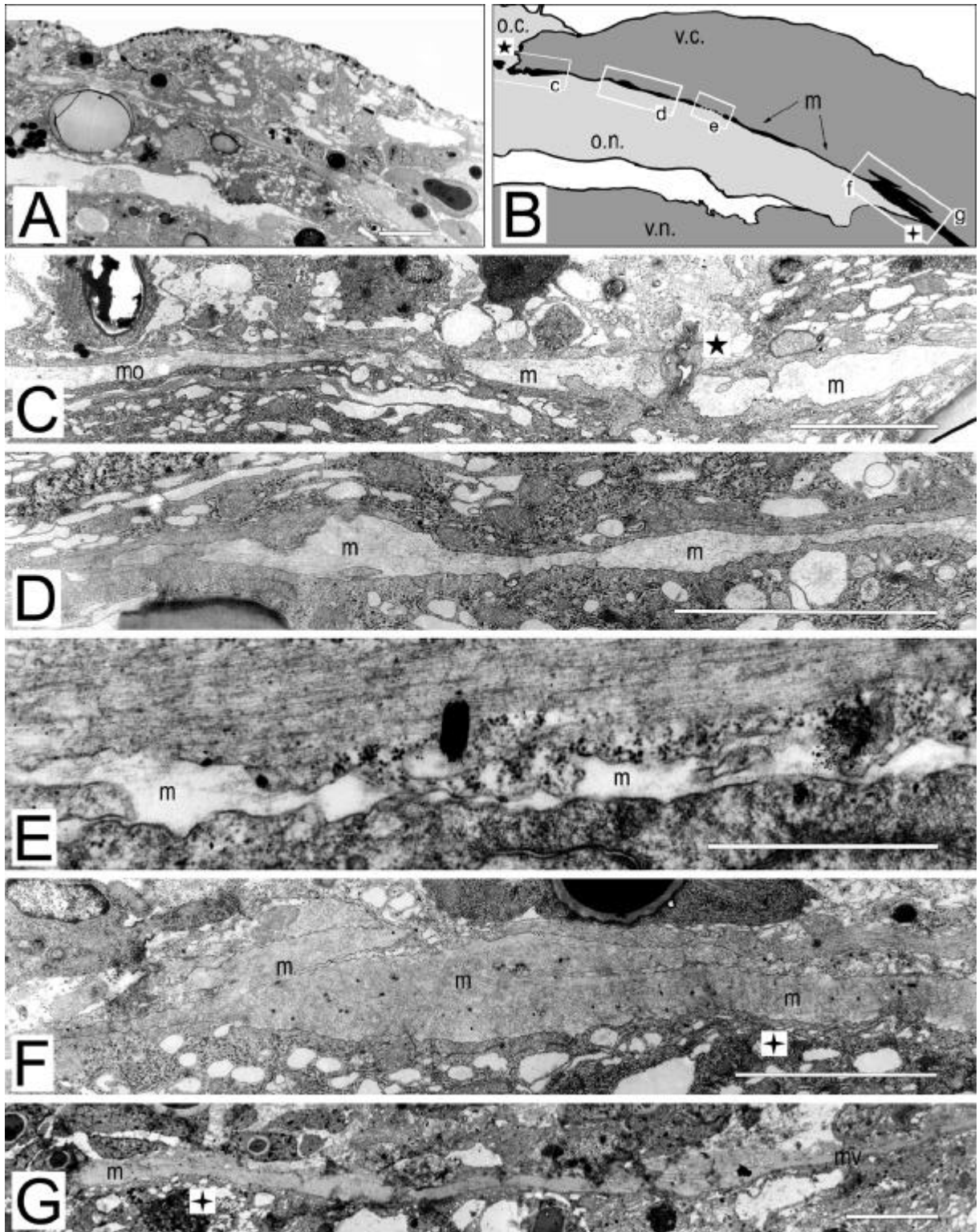


Fig. 4.14 Ultrastructure of the mesoglea in the chimeric region of a 24-hour-old heterograft. (A) – overview of the graft site and (B) – its schematic representation. (o.c.) – *H. oligactis* ectoderm; (v.c.) – *H. vulgaris* ectoderm; (o.n.) – *H. oligactis* endoderm; (v.n.) – *H. vulgaris* endoderm; (m) – chimeric mesoglea. Frames labelled as (c – g) show sites whose enlargements are depicted in (C – G), respectively. Five-pointed and four-pointed starlets mark heterotypic junction sites in

the ectoderm and in the endoderm, correspondingly, thus limiting the chimeric region. Notice how normally medium-thick mesoglea of *H. oligactis* (mo) appears thicker and discontinuous at the heterotypic junction site in ectoderm (C), then alternately thick or elusive in the chimeric region (C and D), thick and branching at the heterotypic junction site in the endoderm (F), and then normally medium-thick (mv) in the *H. vulgaris* tissue (G). Scale bars correspond to 10 μm in (A and G), 5 μm in (C, D, and F) and 1 μm in (E).

4.2.6. Development of heteroaggregates

The contact area between foreign cells is very limited in heterografts. To investigate antagonistic interactions between the cells without such spatial constraints, polyps of both species were dissociated into single-cell suspension, randomly mixed and aggregated together by centrifugation (Gierer et al., 1972). Development of such heteroaggregates was then followed until aggregates transformed to single polyps.

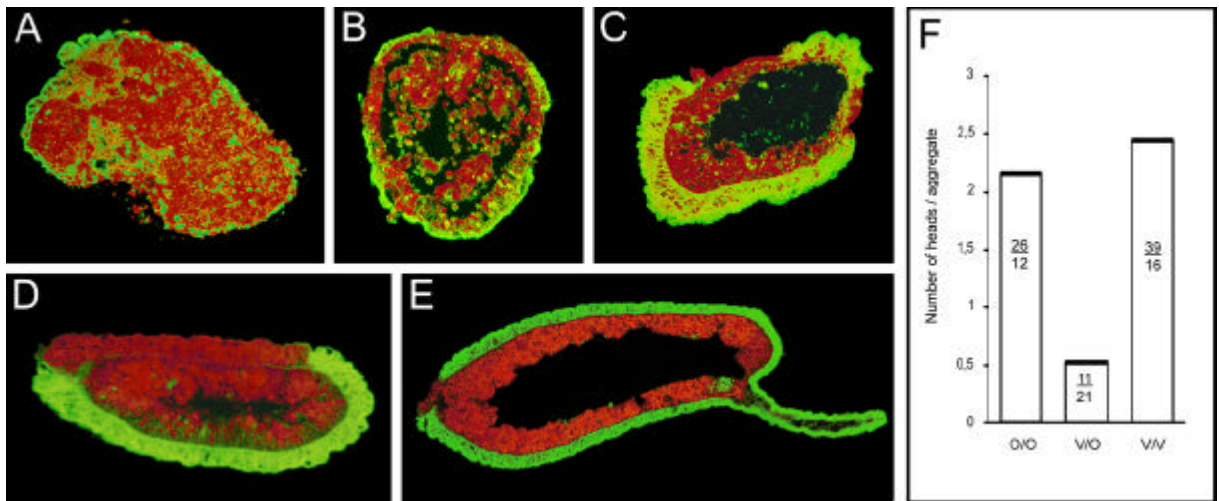


Fig. 4.15 Heteroaggregates slowly develop to ecto-/ endodermal chimeric polyps. (A – E) *H. vulgaris* – *H. oligactis* heteroaggregates 10 hrs, 24 hrs, 7 days, 21 days, and 28 days after reaggregation, respectively. *H. vulgaris* cells are stained with species-specific antiserum visualized with FITC-labelled secondary antibody (green). Unlabelled cells of *H. oligactis* are counterstained with toluidine blue appearing red. **(F)** – histogram indicating number of head structures per aggregate (numbers in columns) on day 7 after reaggregation. (o/o and v/v) correspond to control homoaggregates from *H. oligactis* or *H. vulgaris* alone, respectively. (v/o) corresponds to heteroaggregates.

To monitor behaviour of cells and tissues of both species in such heteroaggregates, aggregates at different stages of development were fixed, embedded in paraffin and sectioned. Sections were then stained with species-specific antibodies.

In *H. vulgaris* – *H. oligactis* heteroaggregates as well as in control homoaggregates, cells of both species quickly sorted according to ectodermal and endodermal layers (Figure 4.15 A – E). In heteroaggregates epithelial cells of each species additionally sorted according to species identity so that as soon as 24 hours after reaggregation clusters of cells of each species were clearly observed (Figure 4.15 B – D). This suggested that cells of the two species have different adhesive properties.

Only a few *H. vulgaris* cells could be found in the endoderm of heteroaggregates 24 hours after reaggregation. Clusters of ectodermal cells of both species grew in size during the development of heteroaggregates (Figure 4.15 B - D). Resultant polyps had an external appearance of *H. oligactis*. However, histological investigation has shown that they were in fact chimeric being comprised of *H. oligactis* endoderm and *H. vulgaris* ectoderm. Only one of the five aggregates examined 28 days after reaggregation proved to be built completely by epithelial cells of the one species, *H. oligactis*. Species identity of epithelial cells in each heteroaggregate was confirmed by staining with species-specific antibody against each species.

Thus cells of these species quickly developed chimeric state with different species in the ectoderm and in the endoderm, similar to heterografts, when being allowed to interact freely.

However, a large portion of holotrichous isorhiza nematocytes characteristic for *H. oligactis* were detected in their tentacles along with those characteristic for *H. vulgaris*. This indicates that interstitial cell lineage of *H. oligactis*, in contrast to epithelial cells, was not affected in ectoderm of the heteroaggregates.

Heteroaggregates appeared more sensitive to the aggregation procedure than control homoaggregates made from the same cell suspensions. They lost more cells during each step of medium dilution. Heteroaggregates showed clear signs of retarded development to polyps. When mature head structures were counted on day 7 after reaggregation, heteroaggregates had four times less of them per aggregate as compared to control homoaggregates (Figure 4.15 F). Eventually all heteroaggregates transformed to polyps, though usually one heteroaggregate gave rise to only one polyp as opposed to 2-3 polyps arising from control homoaggregates. It also took longer for heteroaggregates to digest food. This suggests that heteroaggregates, most of which are chimeric, have serious physiological constraints. One can speculate that physiological constraints in developing

heteroaggregates have the same nature as morphological abnormalities registered for mesoglea in the chimeric region of heterografts.

4.2.7. Role of histocompatibility system in induction of apoptosis

Histocompatibility in terms of allorecognition was previously documented for *Cnidaria* (Humphreys and Reinherz, 1994; Mokady and Buss, 1996). Colonial hydrozoan *Hydractinia* undergoes natural transplantations in the field and has a genetically determined allorecognition system developed to prevent germ cell parasitism (Buss, 1982; Mokady and Buss, 1996).

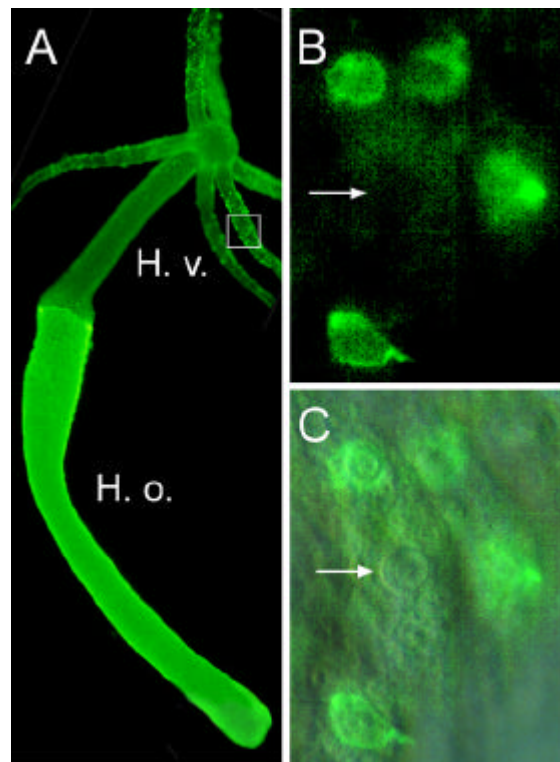


Fig. 4.16 *H. oligactis* nematocytes are not eliminated from *H. vulgaris* tissue. (A) – 1-day-old *H. vulgaris* – *H. oligactis* heterograft stained with species-specific antibodies against *H. oligactis* (green). (B) – enlarged view of a tentacle enclosed in a frame in (A); antibody – positive nematocytes may be seen. (C) – merge of the fluorescence image shown in (B) with a phase contrast view. Arrow indicates an antibody-negative nematocyte of *H. vulgaris*.

Though a histocompatibility system was proposed to operate in *Hydra* heterografts (Bosch and David, 1986), it was surprising because *Hydra* as a solitary animal never undergoes natural transplantation and has no threat of germ cell parasitism. Another

argument raising doubts about the existence of an active histocompatibility system in *Hydra* comes from several observations showing that cells of interstitial cell lineage from one species do not get eliminated in tissues of another *Hydra* species (Lee and Campbell, 1979; Bosch and David, 1986).

Our data confirm this for *H. vulgaris* – *H. oligactis* heterografts. When *H. vulgaris* - *H. oligactis* heterografts were stained with *H. oligactis* – specific antiserum, multiple nematocytes were stained in the tentacles (Figure 4.16). These nematocytes must have been immigrated from the lower *H. oligactis* tissue into the tissue of a foreign species. Surprisingly, they were not eliminated as one would expect in the presence of functional histocompatibility system. Instead, they were embedded into *H. vulgaris* battery cells together with *H. vulgaris* own nematocytes and seemed healthy and ready to operate (Figure 4.16 C).

A highly developed histocompatibility system is able to recognize not only major interspecific differences between tissues but also minor differences between distinct individuals within one species (Rinkevich, 1995). We tested the ability of *H. vulgaris* polyps to induce apoptosis and phagocytosis in response to allogeneic tissues. Along with polyps of the Basel strain of *H. vulgaris*, *H. vulgaris* PA2 strain and a local strain from a lake in Ploen (North Germany) were taken for grafting.

H. vulgaris Ploen strain, though hardly distinguishable from the *H. vulgaris* Basel by morphological criteria, proved most distantly related to the Basel strain by molecular criteria (Figure 4.17). First, polyclonal antiserum primarily generated against membrane fraction of *H. vulgaris* Basel could still produce differential staining of the corresponding portion in *H. vulgaris* Basel – *H. vulgaris* Ploen allografts (Figure 4.17 A). This indicates that the two strains are to a certain extent different on the protein level. Genomic differences between the three *H. vulgaris* strains and *H. oligactis* were analysed by the RAPD analysis (see Material and Methods). Figure 4.17 B demonstrates that the banding pattern produced in PCR amplification with OPA-4 decamer primer is nearly identical for the Basel and PA2 strains of *H. vulgaris*. However, the pattern produced on the template of genomic DNA from *H. vulgaris* Ploen has little in common with other *H. vulgaris* strains as well as with *H. oligactis*. This implies that the Ploen strain is more distantly related to the Basel strain than the PA2 strain is.

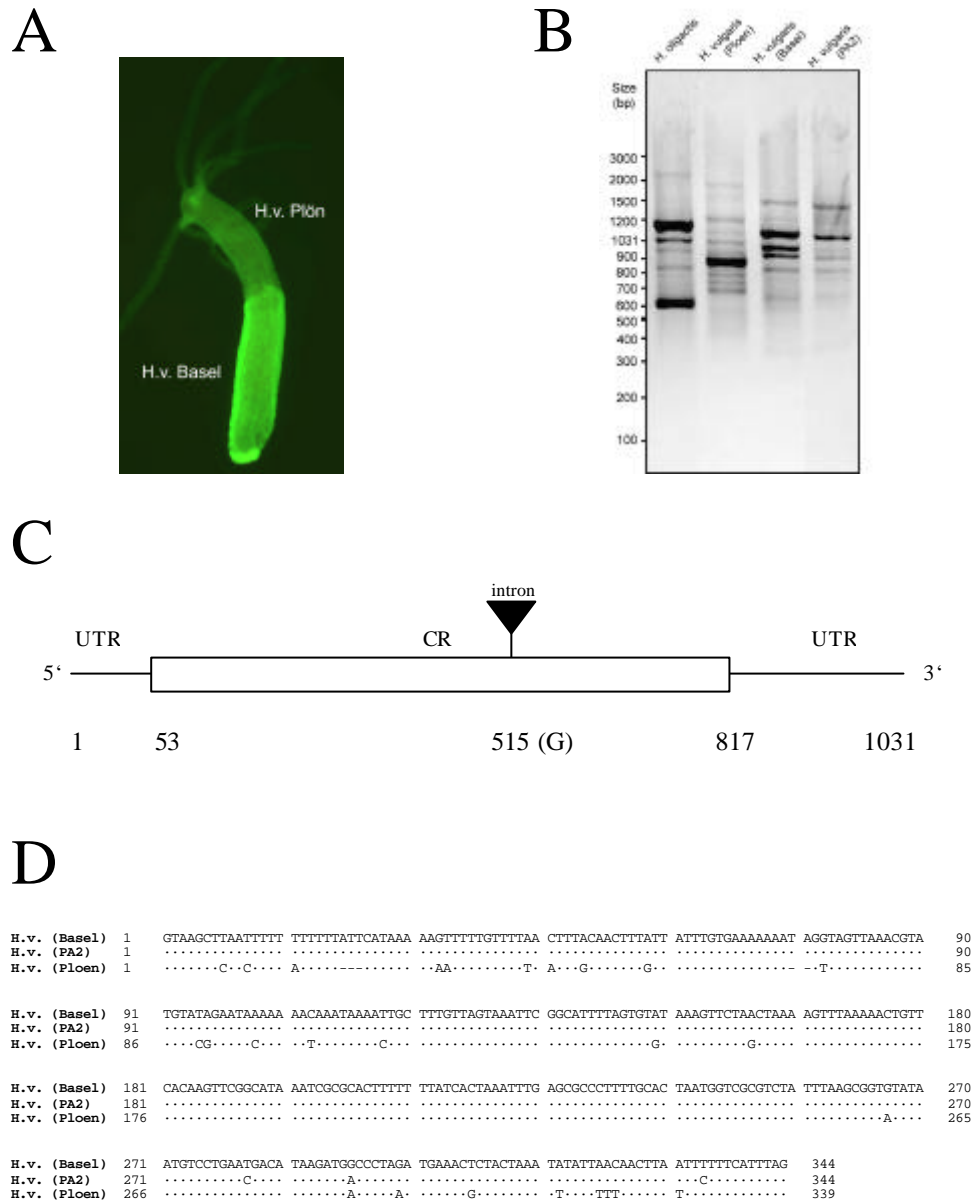


Fig. 4.17 Estimation of allogeneic differences between three strains of *H. vulgaris*. (A) – *H. vulgaris* Ploen – *H. vulgaris* Basel allograft stained with antibodies generated against the Basel strain (green). (B) – RAPD analysis of genomic DNA from *Hydra* strains indicated above the picture. Molecular size in base pairs is indicated at the left side of the figure. (C) – Schematic representation of the cDNA of Cnox-2 gene from *H. vulgaris*. UTR, - untranslated regions; CR, - protein coding region; numbers below the scheme indicate positions of nucleotides along the sequence; the analysed intron was placed after the guanine 515. (D) – Multiple alignment of the Cnox-2 intron sequences from the three strains of *H. vulgaris*. Dots indicate bases identical to that in the Basel strain; dashes indicate gaps inserted by the alignment programme.

To estimate the phylogenetic distance between these strains quantitatively, a piece of a noncoding sequence was isolated for all *H. vulgaris* strains by PCR. As no

histocompatibility alleles are known for *Cnidaria* so far which would be the most appropriate for such comparison, an intron sequence from a randomly chosen gene, a homeobox gene *Cnox-2*, was taken for analysis. Figure 4.17 C graphically demonstrates the position of the intron positioned after guanine 515 and thymine 516 according to the published cDNA sequence of the *Cnox-2* gene of *H. vulgaris* (Gauchat et al., 2000). The intron sequence of *H. vulgaris* strain comprised 344 bases, the same as *H. vulgaris* PA2. *H. vulgaris* Ploen intron sequence comprised 339 bases and contained 9% nucleotide substitutions when compared to the *H. vulgaris* Basel strain (Figure 4.17 D). Only 1% nucleotide differences were estimated for the Basel and PA2 strains. No product could be amplified from *H. oligactis* with the same primers. This suggests even greater differences in the nucleotide sequence of the *Cnox-2* gene between *H. vulgaris* and *H. oligactis*.

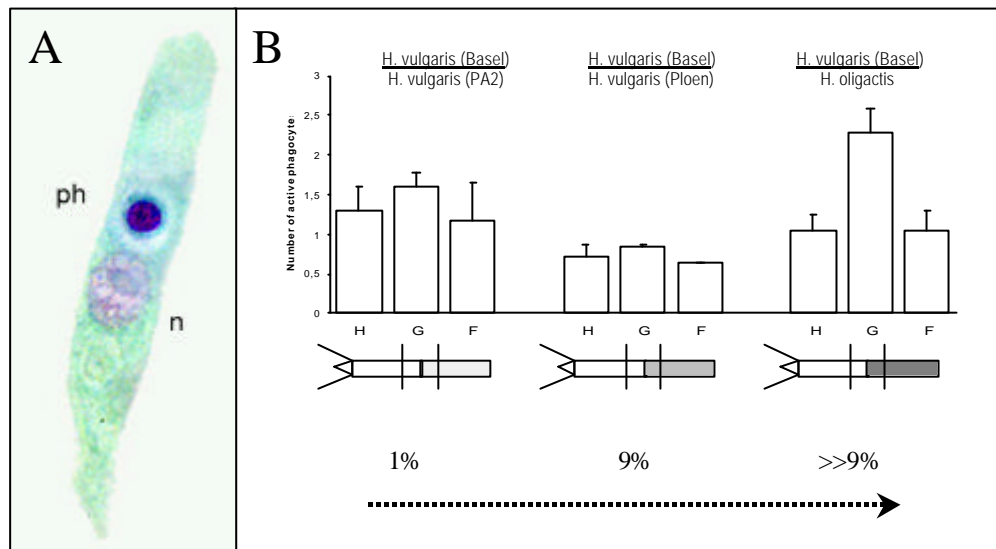


Fig. 4.18 No evidence for the presence of allorecognition in *Hydra*. (A) – a representative cell referred to as an active phagocyte. ph, - phagocytic vacuole containing nucleated cellular material; n, - the nucleus proper of the epithelial cell. (B) – histogram showing the number of active phagocytes in the head (H), graft (G) and foot (F) regions of graft combinations indicated above each group. Numbers in per cent and the arrow are to emphasize that only when strain differences exceed beyond 9% on nucleotide level increase in the level of phagocytosis was registered.

Allogeneic grafts between *H. vulgaris* Basel polyps and polyps of two other strains were prepared. The next day these grafts were cut into three parts - head, graft region and the foot – and at least ten pieces of each segment were macerated together. Maceration preparations were stained by Feulgen method. Epithelial cells containing nucleated cell material in a phagocytic vacuole were considered as active

phagocytes (Figure 4.18 A). Number of active phagocytes was estimated as a percentage from 600 – 1000 epithelial cells counted in total for each slide. Results are shown in Figure 4.18 B.

While in *H. vulgaris* – *H. oligactis* interspecific grafts the number of active phagocytes was twice as high in the graft region than in the head and in the foot, no such increase in phagocytosis was found for allogeneic grafts despite significant allelic differences between the Ploen and two other *H. vulgaris* strains. The number of active phagocytes in all three segments in *H. vulgaris* Basel – *H. vulgaris* PA2 allografts was higher than in the other allogeneic combination. This is probably to be explained by the fact that polyps of the PA2 strain are generally more prone to production of testes, and, as shown previously, many sperm precursors undergo apoptosis and phagocytosis. In one experiment 10 Basel (apical) – PA2 (basal) and 10 PA2 (apical) – Basel (basal) allografts were followed for one month period after transplantation. No polyps carried testes at the moment of grafting. Interestingly, 6 of 10 polyps developed testes in the Basel portion of Basel – PA2 allografts at day 11 after grafting and all 10 at 23rd day after grafting. In allografts with the opposite species orientation only 4 of 10 polyps developed testes at day 23 after grafting. It is tempting to speculate that tissue of the PA2 strain induced testes formation in adjacent tissue of the Basel strain. Alternatively, interstitial sperm precursors of PA2 strain might immigrate into tissues of the Basel strain and induce testes formation. Unfortunately, it is difficult to assess strain identity of sperm precursors in those testes. However, it was the only noticeable interaction between allogeneic tissues in allografts registered thus far. No evidence of allorecognition and induction of apoptosis and phagocytosis have been found in *Hydra* allografts. This implies that histocompatibility system in *Hydra*, when present, is rather poorly developed and some non-immune mechanisms cause induction of apoptosis in the chimeric graft region in interspecific grafts.

5. Discussion

5.1. Role of epithelial cells and apoptosis in Hydra spermatogenesis

In mammalian spermatogenesis, germ cell death is used to remove defective sperm precursor cells (Yin et al., 1998) and to maintain a critical cell number ratio in the testis (Rodriguez et al., 1997). This work presents evidence that this function of apoptosis appears to be conserved across the animal phyla by showing that apoptosis also occurs during spermatogenesis in *Hydra*, a member of the most basal eumetazoan animal phylum *Cnidaria*.

5.1.1. Detection of cell death during spermatogenesis

By Feulgen and acridine orange staining large numbers of sperm precursor cells could be observed which apparently undergo apoptosis in testes. Apoptosis in testes was confirmed by using a TUNEL assay, which identified nuclei with clear signs of DNA fragmentation, a hallmark of apoptosis. Interestingly, the use of the three independent methods to detect apoptotic sperm precursors revealed significant differences. TUNEL staining revealed fewer apoptotic sperm precursors than acridine orange staining (compare figure 4.2 C and E with figure 4.3). This could indicate that in *Hydra* acridine orange identifies all cells and cell fragments which are contained in phagocytic vacuoles while only the TUNEL assay specifically labels cells that are undergoing apoptosis, independent of whether the cells are in vacuoles or not. Alternatively, consistent with the idea that DNA fragmentation is a rapid process, this difference in the staining pattern could indicate that the acridine orange positive phase of apoptosis is longer than the TUNEL positive phase. Another explanation would be that acridine orange positive vacuoles inside epithelial cells represent so-called „residual bodies“, cytoplasmic remainders after spermatozoa are completely formed out of spermatids. In rat testes such cytoplasmic remainders contain apoptotic markers and are subject to phagocytosis by adjacent Sertoli cells (Blanco-Rodriguez and

Martinez-Garcia, 1999). Obviously, such apoptotic bodies would be TUNEL negative.

5.1.2. Function of programmed cell death during spermatogenesis in *Hydra*

In contrast to oogenesis, cell death and phagocytotic activity has never before been observed in *Hydra* spermatogenesis. What is the function of programmed cell death during spermatogenesis in *Hydra*? Sperm precursors in contrast to growing oocytes do not feed on adjacent cells to reach their final size. Since the function seems not to be related to nutrition, there are two major roles for cell death during spermatogenesis. First, apoptosis of sperm precursors and their removal by epithelial cells may be used as “quality control” to eliminate defective sperm. Second, programmed cell death may also be used to achieve the precise homeostasis of each germ cell type in the mature *Hydra* testis. Apoptosis may contribute to maintain a critical cell number ratio between differentiating spermatogonia and epitheliomuscular cells as well as between sperm precursors at different stages of spermatogenesis (Print and Loveland, 2000). Our observation that very few or no apoptotic cells could be found in early testes before the nipple is formed (Figure 4.4) supports the latter point of view. This could mean that, as long as only a few fully differentiated spermatozoa are present in a testis, no signal is sent to spermatogonial cells to suppress their proliferation. But as soon as the distal part of the testis becomes filled with mature sperm, apoptosis is induced in sperm precursors at earlier stages to reduce overall sperm production. In line with this hypothesis is the fact that these are sperm precursors at earlier stages of spermatogenesis that comprise the majority of TUNEL positive cells in *Hydra* testes (Figure 4.5). On the other hand, spermatocytes are the cells undergoing meiosis and are most vulnerable to recombination defects what could result in induction of apoptosis in particular at this stage as a quality control mechanism (Odorisio et al., 1998).

An additional argument favouring the role of apoptosis as a mechanism regulating cell number ratio rather than quality control comes from studies on spermatogenesis in the nematode *C. elegans*. In contrast to many other animals, in *C. elegans* sperm production (but not oogenesis) does not necessarily involve apoptotic death of sperm precursors (Gumienny et al., 1999), although

occasionally damaged cells still can be eliminated by apoptosis (Gartner et al., 2000). One can speculate, that cell proliferation and differentiation in *C. elegans* is as precisely orchestrated during spermatogenesis as it is during the embryonic development. Therefore in *Hydra*, in contrast to *C. elegans*, apoptosis may have to be necessary to adjust cell number ratio of sperm precursors. Further massive apoptosis found in degrading testes (Figure 4.4 C) suggests that apoptosis functions here to remove a no-more necessary structure.

Experimental evidence for the precise function of apoptosis during spermatogenesis in *Hydra* could now be obtained by silencing of genes (Lohmann et al., 1999), which are critical for the execution of apoptosis.

5.1.3. Epithelial cells play an active role in *Hydra* spermatogenesis

Hydra as a member of the basal eumetazoan phylum *Cnidaria* has a limited number of different cell types. Ectodermal epithelial cells are multifunctional cells and as “epitheliomuscular cells” are responsible for motility. In addition, epithelial cells also appear to control morphogenesis (Bosch, 1998) as well as the phagocytic removal of “excess” cells (Bosch and David, 1984; Fujisawa and David, 1984; Bosch and David, 1986). Until now there was no evidence that epithelial cells were also actively involved in spermatogenesis, which was thought to be an autonomous function of interstitial stem cell differentiation. The role of ectodermal epitheliomuscular cells in spermatogenesis was confined to forming the outer wall of the testes (Tardent, 1985). In contrast to that view, our data clearly show that ectodermal epithelial cells play an active role during spermatogenesis as phagocytes.

Recent evidence from genetically modified mice indicates that control of male germ cell survival is mediated by signals derived from the Sertoli cells such as growth factors, paracrine factors, nutrients, and physical contact (reviewed in Griswold, 1998; Print and Loveland, 2000). Sertoli cells also remove apoptotic germ line cells per phagocytosis. Based on our observations it is tempting to speculate that in *Hydra* testes the epitheliomuscular cells, which are closely adjacent to the developing spermatozoa (Figure 2.1), function like Sertoli cells by controlling the microenvironment of the developing interstitial sperm precursor

cells and sequestering the sperm precursor populations into environmentally distinct compartments. The view that epithelial cells in *Hydra*, in addition to their role as phagocytes, produce specific factors, which are necessary for interstitial cell differentiation, is supported by the recent observation (Takahashi et al., 2000) that *Hydra* epithelial cells directly influence the nerve cell differentiation pathway by secreting PW peptides.

The scheme summarizing presumable functions of epithelial cells in *Hydra* spermatogenesis in comparison with Sertoli cells in mammalian spermatogenesis is presented in Figure 5.1.

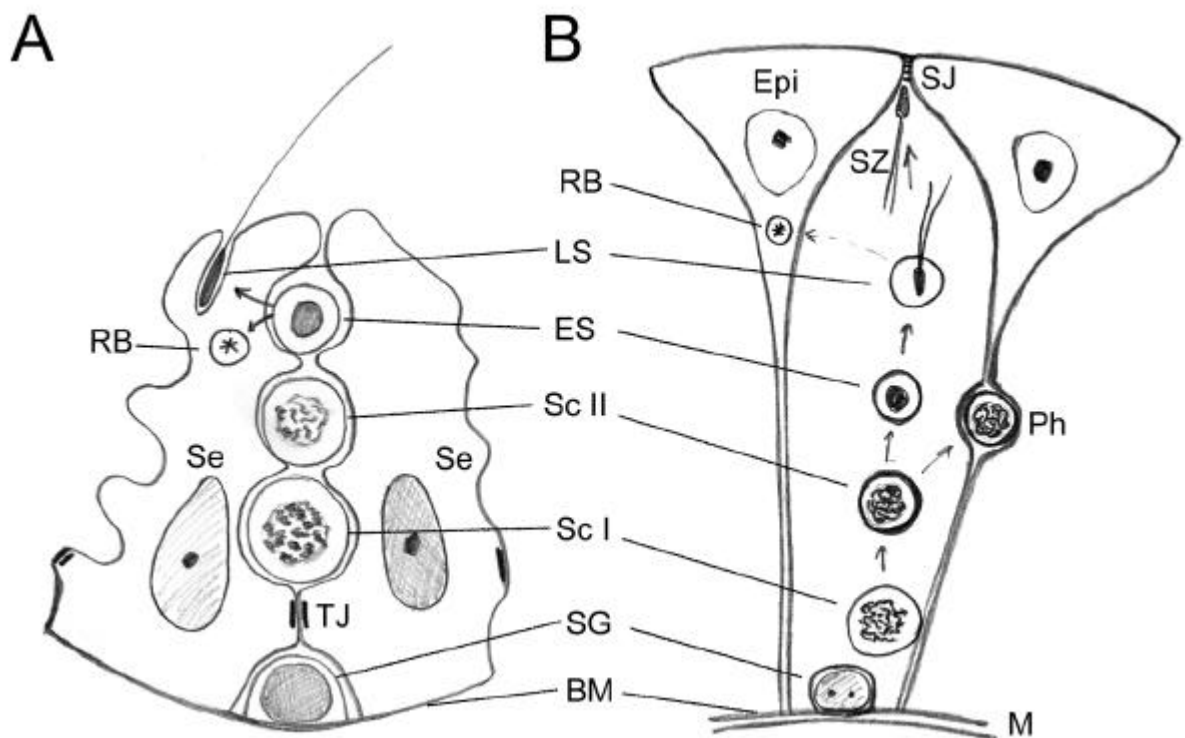


Fig. 5.1 Structural relationship between somatic and germ line cells during spermatogenesis in mammals (A) and *Hydra* (B). In mammals Sertoli cells (Se), being interconnected through tight junctions (TJ), sequester spermatogonial cells (SG) to the basal compartment and cells at the following spermatogenic stages to the adluminal compartment. Residual bodies (RB) remaining from the early spermatids (ES) are phagocytosed by Sertoli cells. In *Hydra* sperm precursors are similarly segregated within compartments built by ectodermal epithelial cells (Epi) sealed by septate junctions (SJ). Long epithelial protrusions are in close contact with sperm precursors and can phagocytose cells undergoing apoptosis (Ph). Other abbreviations: Sc I, - primary spermatocytes; Sc II, - secondary spermatocytes; LS, - late spermatids; SZ, - spermatozoa; M, - mesoglea; BM, - basement membrane

5.2. Apoptosis in interspecific grafts of Hydra

Defective interaction between different cells and extracellular matrix can cause induction of apoptosis (Barres et al., 1993; Frisch and Francis, 1994). Our data suggest that this feature of multicellular animals is well conserved down to the simple diploblastic animal *Hydra*.

5.2.1. Evidence for apoptosis in interspecific grafts

The presence of apoptotic cells in *H. vulgaris* – *H. oligactis* interspecific grafts was clearly shown by using vital acridine orange staining and TUNEL assay (Figure 4.7). As during spermatogenesis, fewer cells were positively stained by TUNEL method than with acridine orange in heterografts, probably because the TUNEL positive phase is shorter than the acridine orange positive one. Cells with fragmented DNA (TUNEL assay) and phagocytosed cells with low pH (acridine orange positive cells) were all restricted to *H. vulgaris* portion of the grafts close to the grafting site irrespective of species orientation along the body axis. This is consistent with the previous finding (Bosch and David, 1986), also confirmed in this study, that there is increase in phagocytosis in the graft region in heterografts as revealed by Feulgen staining on macerates. Phagocytosis is tightly linked to apoptosis and usually follows it (see Introduction). In addition some phagocytosed cells in such preparations demonstrate highly condensed chromatin structure as characteristic for apoptotic cells (Figure 4.18 A). This suggests that acridine orange and TUNEL positive cells revealed in whole mount stainings visualize cell death found previously on maceration preparations.

5.2.2. Structural incompatibility as the likeliest cause of apoptosis

What caused induction of apoptosis in heterografts? Increased level of apoptosis at the graft border occurred only when tissue portions of quite different species, *H. vulgaris* and *H. oligactis*, were brought in physical contact by grafting. Two alternative mechanisms can be suggested to be able to induce apoptosis in this situation. First, death of cells of either species can be caused by active cytotoxic

reaction as defence against non-self. This possibility will be discussed later in chapter 5.2.3. Second, cell death at the species border can occur due to inability of cells of one species to respond in a proper way to survival signals provided by cells of another species. Our data favour the latter idea. Argumentation follows.

It turned out that the area in heterografts, where apoptotic cells were found, is in fact chimeric in a way that here ectoderm of one species, *H. vulgaris*, is underlied by endoderm of another species, *H. oligactis* (compare Figures 4.7 A, D with Figure 4.13 C, H and Figure 4.7 B, E with Figure 4.13 E, J). This must have resulted from differential movement of one epithelial layer relative to the other. During this process endoderm of *H. vulgaris* is detached from the mesoglea and substituted by *H. oligactis* endoderm (Figure 4.12). It cannot be excluded that *H. vulgaris* ectoderm to a certain extent also contributes to creating chimeric area by moving in the opposite direction. The phenomenon of differential movement of epithelia was previously described for *H. viridis* and *H. vulgaris* (Campbell, 1974; Wanek and Campbell, 1982). However, in the latter study epithelial shift of only 0.5 mm in average length could be best achieved when polyps were grafted in the upper gastric region. Besides, the ectodermal layer moved quicker distally than the endodermal layer. In case of *H. vulgaris* – *H. oligactis* heterografts the direction of epithelial movement appeared independent of position of the grafting site relative to the body axis. Instead, it depended solely on that in which position, apical or basal, *H. vulgaris* portion was placed in a graft. This rather means that some intrinsic differences exist between epithelial cells of the two species, which reproducibly lead to substitution of *H. vulgaris* endoderm by that of *H. oligactis*.

Observations on developing heteroaggregates provide evidence that formation of interspecific ecto- / endodermal chimeric regions is a general phenomenon inherent for this species pair. Initially randomly mixed, cells of both species sorted out according to the outer or inner epithelial layers and according to species identity (Figure 4.15). However, cells of both species demonstrated quite distinct behaviour as aggregates developed. First, most of the *H. vulgaris* cells disappeared from the endodermal layer early during aggregate development (Figure 4.15 B). Second, cells of each species clustered separately from each other in the ectoderm, but at the end only *H. vulgaris* epithelial cells remained (Figure 4.15 E). Thus, though starting conditions were quite different in

heterografts and in heteroaggregates, the resultant chimeric combination was strikingly similar.

Such cell sorting in heteroaggregates can be partly explained by the theory of differential cell adhesiveness (Townes and Holtfreter, 1955; Steinberg, 1970). This theory predicts that two cell types with different strength of homotypic intercellular adhesion and intermediate strength of heterotypic adhesiveness would build a bilayered sphere, with inner layer comprised by cells with highest affinity. Implementation of this theory to cell sorting in *Hydra* aggregates is justified by experimental measurement of adhesive forces between *Hydra vulgaris* endodermal and ectodermal cells being estimated as 50 pN and 30 pN, respectively (Sato-Maeda et al., 1994). Thus, in heteroaggregates *H. vulgaris* endodermal cells could sort to the inner cell mass and be expelled from the aggregate tissue, if their adhesion is stronger than that between *H. oligactis* cells. On the other hand, Kishimoto and colleagues (1996) argued that a substantial signalling exchange between ectodermal and endodermal cells has to be involved to form bilayered body wall in *Hydra*, in addition to differential cell adhesion. In heteroaggregates, *H. oligactis* endoderm might outcompete *H. vulgaris* endoderm due to more effective interaction with the ectodermal layer even when ectoderm is comprised by *H. vulgaris* epithelial cells. By analogy, *H. vulgaris* ectoderm could gradually outcompete *H. oligactis* ectoderm. Whether or not the same mechanisms govern formation of chimeric region in heterografts and in heteroaggregates is not known, but the result is nevertheless comparable. Ultrastructural study of the chimeric region in heterografts revealed morphologically abnormal mesoglea separating *H. vulgaris* ectoderm and *H. oligactis* endoderm (Figure 4.14). Mesoglea, normally a 0.5 – 2.0 μm thick layer of extracellular matrix, was alternately unusually thick (Figure 4.14 F) or nearly absent (Figure 4.14 E) at several places in the chimeric region but not in adjacent normal tissues. Mesoglea is comprised by several types of macromolecules such as laminins, collagens, heparan sulfate proteoglycans and fibronectin-like molecules (Sarras et al., 1991). These molecules play essential role in cell proliferation, cell migration and morphogenesis in *Hydra* (reviewed in Sarras and Deutzmann, 2001). Morphologically abnormal condition of mesoglea in the chimeric region implies that at least some of its functions might be also affected. Consistent with the idea of functional disability of the mesoglea, chimeric

aggregates revealed remarkable retardation of development (Figure 4.15 F) similar to what happens when biosynthesis of mesogleal proteins is perturbed (Sarras et al., 1993). It is tempting to speculate that some cells in the chimeric region determined to proliferate, or migrate, or to perform a morphogenetic function would undergo apoptosis due to the lack of permitting signal from mesoglea. The fact that mesoglea is not completely destroyed in the chimeric region of heterografts may explain why not all cells undergo apoptosis in this region. On the other hand, not every cell's survival may be strictly dependent on mesoglea.

Epithelial cell survival is frequently dependent on anchorage via integrins to extracellular matrix molecules such as laminin, fibronectin and collagen in higher animals (Frisch and Screaton, 2001). Cell death resulted from the loss of integrin anchorage is termed anoikis (see Introduction). No anoikis mediated cell death was reported for *Cnidaria* so far. However, this mechanism is likely to exist as survival of many cnidarian cells in culture was found to be dependent on attachment to extracellular matrix substrates (Schmid et al., 1999). In *Hydra*, laminin participates in formation of basement membranes of both epithelial layers but is secreted exclusively by the endoderm (Sarras et al., 1994). Conversely, collagen-I comprises fibrillar matrix of the mesoglea and arises from the ectoderm (Deutzmann et al., 2000). Production of collagen-I by ectodermal epithelial cells is preceded by and dependent on the production of laminin by endodermal epithelial cells (Sarras and Deutzmann, 2001). One can speculate that these components of extracellular matrix may be structurally different between distantly related species such as *H. vulgaris* and *H. oligactis*, and this difference could be great enough to prevent their appropriate recognition by the cells of the counterpart species and provoke cell death via anoikis. Alternatively, incompatible species-specific differences between certain ECM molecules could lead to substantial collapse of the mesoglea structure and induction of apoptosis in cells whose survival depends on it as discussed above. Species-specific differences in signalling molecules other than extracellular matrix could also result in apoptosis of certain cells in the chimeric zone. Unfortunately, functional significance of species-specific differences in neither but one *Hydra* molecules was studied so far (Brennecke et al., 1998). It would be interesting to test this hypothesis when appropriate tools become available.

5.2.3. Potential role of direct non-self recognition in induction of apoptosis

Direct non-self recognition followed by cytotoxic reaction was previously proposed to be the cause of increased level of phagocytosis at the graft border in *Hydra* heterografts (Bosch and David, 1986). Non-self recognition in terms of xeno- and allorecognition was extensively studied in vertebrate and in some invertebrate animals (Abbas et al., 1994; Humphreys and Reinherz, 1994). Many animals were shown to mount considerable cytotoxic reactions against cells and tissues having minimal genetic differences to the host cells (Abbas et al., 1994).

Studies on vertebrates' immune system revealed that histocompatibility is determined mainly by only a few genes such as MHC antigens and T cell receptor molecules. No such genes were found in invertebrates so far. However, genetic studies on protochordate *Botryllus* (Scofield et al., 1982) and hydrozoan cnidarian *Hydractinia* (Mokady and Buss, 1996) have estimated that only one genetic locus is responsible for allorecognition reactions in these species. These genes harbour hundreds of alleles and such a high polymorphism is the basis for high discriminative capacity of these animals (Takahata et al., 1992).

High polymorphism in MHC loci in vertebrates is essential for the recognition of a broad spectrum of pathogens and is strongly supported by a form of balancing selection (Hughes and Yeager, 1998). Many invertebrates harbouring highly discriminative allorecognition system are colonial and prone to frequent allogeneic confrontation naturally in the field. Chimerism between unrelated colonies was experimentally shown to be disadvantageous for one of both partners (Rinkevich, 1995; Stoner and Weissmann, 1996). Leo Buss proposed that highly discriminative allorecognition system could evolve in such animals to prevent somatic and germ cell parasitism occurring when respective cells migrate from one colony into another after colony fusion (Buss, 1982).

Hydra is a solitary freshwater polyp and has no means to directly confront allogeneic or xenogeneic tissues naturally in the field. This implies that selection pressure supporting allorecognition system in colonial cnidarians would be much reduced in *Hydra*. Apparently, *Hydra* also does not use its stinging cells to fight for the living space like solitary sea anemones do (Lubbock, 1980). Thus, one should expect that *Hydra* would be unable to mount significant cytotoxic

responses against genetically distinct cells. Nevertheless, it cannot be completely excluded that to some extent *Hydra* did retain this capacity.

We tested allorecognition potential of *Hydra* by surgically grafting polyps of genetically distinct *H. vulgaris* strains. Genetic differences were estimated on protein and genomic levels as well as on nucleotide sequence level (Figure 4.17). As no „histocompatibility genes“ are known for invertebrates so far, we took a noncoding piece of a random gene, *cnox-2* homeobox gene, as a reference. *H. vulgaris* PA2 and *H. vulgaris* Ploen strains were taken for grafting experiments as strains, respectively, the least and the most diverged ones from the standard *H. vulgaris* Basel strain. No rejection or increased incidence of phagocytosis could be observed in neither of the allografts (Figure 4.18). This suggests that *Hydra* has in fact highly reduced capacity for allorecognition.

H. vulgaris and *H. oligactis* are phylogenetically very diverged species (Percell et al., 2001) and their cells, therefore, might react against each other in heterogeneic grafts. However, induction of apoptosis and phagocytosis in the graft region observed in such grafts does not seem to result due to non-self recognition reaction because of the following reasons. *Hydra* epithelial cells can migrate only together with the whole epithelial sheet (Campbell, 1974). Thus apoptotic cells should be restricted only to the border line of the graft where epithelial cells of two species confront each other, what was not the case (Figure 4.7). The only freely migrating cells in *Hydra* able to enter foreign epithelium are cells of interstitial lineage. We show that these cells do immigrate into the foreign tissue, but do not get eliminated there as would be expected (Figure 4.16). One could speculate that *H. oligactis* tissue exerted a long-distance influence on adjacent tissue of *H. vulgaris* by secreting some toxic substances. However, no evidence for such interactions was found in *Hydra* to date.

Instability of *Hydra* interspecific grafts was sometimes considered as demonstration of immune rejection (Du Pasquier, 1974). Opposing view was that such instability is determined by poor cell adhesion between foreign cells leading to inability to preserve the integrity of the epithelial body wall (Campbell, 1973). Consistent with the latter idea, our ultrastructural studies of the heterotypic junctions in the epidermis revealed highly irregular septate desmosomes, which could impossibly fulfil their function to isolate underlying tissues from

hypoosmotic surroundings (Figure 4.11) and would be the most likely cause of heterograft instability.

Taken together, this suggests that histocompatibility system, when present at all, plays a minor role in induction of apoptosis in *Hydra* heterografts.

6. Summary

The freshwater cnidarian polyp hydra is the simplest organism hitherto known to possess components of the apoptotic machinery. However, little is known about the functions of apoptosis in hydra.

Here I present evidence for the role of apoptosis in hydra spermatogenesis and histocompatibility. Cells undergoing programmed cell death were detected by vital acridine orange staining and TUNEL assay.

Sperm precursors were shown to undergo apoptosis mostly at early stages of spermatogenesis. Role of apoptosis as a mechanism to adjust the number of sperm precursors at different stages of spermatogenesis is discussed. Surprisingly, epithelial cells, previously thought to function merely as physical support of the testes, were found to play an active role during spermatogenesis as phagocytes. Similar to Sertoli cells in mammals they may provide signals to differentiating sperm.

Apoptosis was also found to be induced at the graft border in hydra interspecies grafts. Though self / non-self recognition was proposed previously to play a crucial role in such heterografts, no evidence for existence of allorecognition system was found. Hydra seems to have lost this system secondarily during evolution. Ultrastructural studies of the graft site revealed morphologically disturbed mesoglea separating *H. vulgaris* ectoderm and *H. oligactis* endoderm. Apoptosis is proposed to result due to incompatibility between tissues of these two species. Role of incompatibility mechanisms such as anoikis or inability to properly recognize survival signals from a different species is discussed.

6. Zusammenfassung

Der Süßwasserpolyt *Hydra* ist der einfachste Organismus der über Komponenten der Apoptosemaschinerie verfügt. Jedoch ist wenig über die Funktion des programmierten Zelltodes in *Hydra* bekannt.

In dieser Arbeit präsentiere ich Hinweise für die Rolle der Apoptose in der Spermatogenese und der Gewebekompatibilität. Die Zellen, die den programmierten Zelltod durchlaufen, wurden mit Hilfe der Acridinorange-Färbung und dem TUNEL - Assay detektiert.

Es konnte gezeigt werden, dass Spermiovorläuferzellen hauptsächlich in den frühen Phasen der Spermatogenese Apoptose durchlaufen. In diesem Zusammenhang wird die Rolle der Apoptose zur Kontrolle der Anzahl von Spermiovorläuferzellen zu verschiedenen Spermatogenesestadien diskutiert. Überraschenderweise konnte gezeigt werden, dass die Epithelzellen eine aktive Rolle während der Spermatogenese als Phagozyten spielen. Darüber hinaus könnten sie, wie die Sertoli Zellen bei den Säugetieren, über Signalinteraktionen die Differenzierung der Spermien beeinflussen.

Apoptose konnte auch an der Transplantationsgrenze in Transplantaten zwischen zwei Hydraarten nachgewiesen werden. In früheren Experimenten wurde diese Beobachtung mit dem Umstand der Selbst/Nichtselbsterkennung zu erklären versucht. In der vorliegenden Arbeit konnten keine Beweise für das Vorhandensein eines Selbsterkennungssystems in *Hydra* gefunden werden. Es scheint, als ob *Hydra* während ihrer Evolution dieses System sekundär verloren hat. Ultrastrukturelle Untersuchungen des Transplantationsbereiches deckten eine morphologisch veränderte bzw. gestörte Mesoglea auf, welche das *H. vulgaris* Ektoderm vom *H. oligactis* Entoderm trennt. Der beobachtete programmierte Zelltod in dieser Region könnte ein Ergebnis von Gewebeinkompatibilitäten zwischen beiden Spezies sein. Gegenstand der Diskussion sind in dem Zusammenhang Mechanismen die zu Inkompatibilität zwischen Geweben führen können, wie z.B. Anoikis oder die Unfähigkeit „Überlebensfaktoren“ der anderen Spezies zu erkennen.

7. References

- Abbas, A.K., Lichtman, A.H. & Pober, J.S. (1994). *Cellular and molecular immunology*. W.B. Saunders Company Philadelphia,
- Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. & Yuan, J. (1996). Human ICE/CED-3 protease nomenclature. *Cell* **87**, 171-171.
- Arendt, D., Technau, U. & Wittbrodt, J. (2001). Evolution of the bilaterian larval foregut. *Nature* **409**, 81-85.
- Barde, Y.A. (1989). Trophic factors and neuronal survival. *Neuron* **2**, 1525-1534.
- Barres, B.A., Hart, I.K., Coles, H.S.R., Burne, J.F., Voyvodic, J.T., Richardson, W.D. & Raff, M.C. (1992). Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* **70**, 31-46.
- Bibb, C. & Campbell, R.D. (1973a). Cell affinity determining heterospecific graft intolerance in hydra. *Tissue Cell* **5**, 199-208.
- Bibb, C. & Campbell, R.D. (1973b). Tissue healing and septate desmosome formation in hydra. *Tissue Cell* **5**, 23-35.
- Blagosklonny, M.V. (2000). Cell death beyond apoptosis. *Leukemia* **14**, 1502-1508.
- Blanco, R. & Martinez, G. (1999). Apoptosis is physiologically restricted to a specialized cytoplasmic compartment in rat spermatids. *Biol Reprod* **61**, 1541-1547.
- Bode, H., Berking, S., David, C.N., Gierer, A., Schaller, H. & Trenkner, E. (1973). Quantitative analysis of cell types during growth and morphogenesis in *Hydra*. *Wilhelm Roux's Arch.Dev.Biol.* **171**, 269-285.
- Bosch, T.C. (1998). Hydra. In *Cellular and Molecular Basis of Regeneration: from Invertebrates to Humans* (Ferretti, P. and Géraudie, J., eds.), Wiley & Sons, Sussex,
- Bosch, T.C. & David, C.N. (1984). Growth regulation in Hydra: relationship between epithelial cell cycle length and growth rate. *Dev Biol* **104**, 161-171.
- Bosch, T.C. & Fujisawa, T. (2001). Polyps, peptides and patterning. *Bioessays* **23**, 420-427.
- Bosch, T.C.G. & David, C.N. (1986). Immunocompetence in Hydra: epithelial cells recognize self-nonsel and react against it. *J Exp Zool* **238**, 225-234.
- Bosch, T.C.G. & David, C.N. (1987). Stem cells of Hydra magnipapillata can differentiate into somatic and germ line cells. *Dev Biol* **121**, 182-191.

- Brennecke, T., Gellner, K. & Bosch, T.C. (1998). The lack of a stress response in *Hydra oligactis* is due to reduced hsp70 mRNA stability. *Eur J Biochem* **255**, 703-709.
- Brien, P. (1966). *Biologie de la reproduction animale*. Masson & Cie Paris,
- Brien, P. & Reniers-Decoen, M. (1951). La gamétogénèse at l'intersexualité chez *Hydra attenuata* (Pall.). *Ann.Soc.Roy.zool.Belg.* **82**, 285-327.
- Buendia, B., Santa, M. & Courvalin, J.C. (1999). Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. *J Cell Sci* **112**, 1743-1753.
- Bullock, A.N. & Fersht, A.R. (2001). Rescuing the function of mutant p53. *Nature Reviews Cancer* **1** , 68-76.
- Bullock, A.N., Henckel, J. & Fersht, A.R. (2000). Quantitative analysis of residual folding and DNA binding in mutant p53 core domain: definition of mutant states for rescue in cancer therapy. *Oncogene* **19**, 1245-1256.
- Buss, L.W. (1982). Somatic cell parasitism and the evolution of somatic tissue compatibility. *Proc Natl Acad Sci U S A* **79**, 5337-5341.
- Campbell, R.D. (1974). Cell movements in *Hydra*. *Amer.Zool.* **14**, 523-535.
- Campbell, R.D. (1976). Elimination of *Hydra* interstitial and nerve cells by means of colchicine. *J Cell Sci* **21**, 1-13.
- Campbell, R.D. (1987). A new species of *Hydra* (Cnidaria: Hydrozoa) from North America with comments on species clusters within the genus. *Zool.J.Linnean.Soc.* **91**, 253-263.
- Campbell, R.D. & Bibb, C. (1970). Transplantation in coelenterates. *Transplant Proc* **2**, 202-211.
- Cikala, M., Wilm, B., Hobmayer, E., Bottger, A. & David, C.N. (1999). Identification of caspases and apoptosis in the simple metazoan *Hydra*. *Curr Biol* **9**, 959-962.
- Clerc, S. & Barenholz, Y. (1998). A quantitative model for using acridine orange as a transmembrane pH gradient probe. *Anal Biochem* **259**, 104-111.
- Coucouvanis, E. & Martin, G.R. (1995). Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. *Cell* **83**, 279-287.
- David, C.N. (1972). Quantitative method for maceration of hydra tissue. *Wilhelm Roux's Arch.Dev.Biol.* **171**, 259-263.
- David, C.N., Bosch, T.C.G., Hobmeyer, B., Holstein, T. & Schmidt, T. (1987). The interstitial stem cells in hydra. In *Genetic regulation of development* (Loomis, W.F., ed.), Alan R. Liss Inc., New York, pp. 389-408.
- David, C.N. & Murphy, S. (1977). Characterization of interstitial stem cells in hydra by cloning. *Dev Biol* **58**, 372-383.

- Davis, L.E., Burnett, A.L. & Haynes, J.F. (1968). Histological and ultrastructural study of the muscular and nervous systems in Hydra. II. Nervous system. *J Exp Zool* **167**, 295-331.
- Day, R.M. & Lenhoff, H.M. (1981). Hydra mesoglea: a model for investigating epithelial cell--basement membrane interactions. *Science* **211**, 291-294.
- De, J., Hoeberichts, F.A., Yakimova, E.T., Maximova, E. & Woltering, E.J. (2000). Chemical-induced apoptotic cell death in tomato cells: involvement of caspase-like proteases. *Planta* **211**, 656-662.
- Deutzmann, R., Fowler, S., Zhang, X., Boone, K., Dexter, S., Boot, H., Rachel, R. & Sarras, M.P. (2000). Molecular, biochemical and functional analysis of a novel and developmentally important fibrillar collagen (Hcol-I) in hydra. *Development* **127**, 4669-4680.
- Deveraux, Q.L. & Reed, J.C. (1999). IAP family proteins--suppressors of apoptosis. *Genes Dev* **13**, 239-252.
- Du, C., Fang, M., Li, Y., Li, L. & Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* **102**, 33-42.
- Du Pasquier (1974). The genetic control of histocompatibility reactions: phylogenetic aspects. *Arch Biol (Liege)* **85**, 91-103.
- Earnshaw, W.C., Martins, L.M. & Kaufmann, S.H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* **68**, 383-424.
- Elangbam, C.S., Qualls, C.W. & Dahlgren, R.R. (1997). Cell adhesion molecules--update. *Vet Pathol* **34**, 61-73.
- Fadok, V.A., Bratton, D.L., Rose, D.M., Pearson, A., Ezekewitz, R.A. & Henson, P.M. (2000). A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* **405**, 85-90.
- Frisch, S.M. & Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* **124**, 619-626.
- Frisch, S.M. & Screaton, R.A. (2001). Anoikis mechanisms. *Curr Opin Cell Biol* **13**, 555-562.
- Fujisawa, T. & David, C.N. (1984). Loss of differentiating nematocytes induced by regeneration and wound healing in Hydra. *J Cell Sci* **68**, 243-255.
- Gartner, A., Milstein, S., Ahmed, S., Hodgkin, J. & Hengartner, M.O. (2000). A conserved checkpoint pathway mediates DNA damage--induced apoptosis and cell cycle arrest in *C. elegans*. *Mol Cell* **5**, 435-443.
- Gauchat, D., Mazet, F., Berney, C., Schummer, M., Kreger, S., Pawlowski, J. & Galliot, B. (2000). Evolution of Antp-class genes and differential expression of

- Hydra Hox/paraHox genes in anterior patterning. *Proc Natl Acad Sci U S A* **97**, 4493-4498.
- Gavrieli, Y., Sherman, Y. & Ben, S. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* **119**, 493-501.
- Gierer, A. (1977). Biological features and physical concepts of pattern formation exemplified by hydra. *Curr Top Dev Biol* **11**, 17-59.
- Gierer, A., Berking, S., Bode, H., David, C.N., Flick, K., Hansmann, G., Schaller, H. & Trenkner, E. (1972). Regeneration of hydra from reaggregated cells. *Nat New Biol* **239**, 98-101.
- Gilbert, S. (2000). *Developmental biology*. Sinauer Associates, Inc. Sunderland, Massachusetts,
- Glucksmann, A. (1951). Cell deaths in normal vertebrate ontogeny. *Biol.Rev.* **26**, 59-86.
- Griswold, M.D. (1998). The central role of Sertoli cells in spermatogenesis. *Semin Cell Dev Biol* **9**, 411-416.
- Gumienny, T.L., Lambie, E., Hartwig, E., Horvitz, H.R. & Hengartner, M.O. (1999). Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. *Development* **126**, 1011-1022.
- Hamon, Y., Broccardo, C., Chambenoit, O., Luciani, M.F., Toti, F., Chaslin, S., Freyssinet, J.M., Devaux, P.F., McNeish, J., Marguet, D. & Chimini, G. (2000). ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. *Nat Cell Biol* **2**, 399-406.
- Hanahan, D. & Weinberg, R.A. (2000). The hallmarks of cancer. *Cell* **100**, 57-70.
- Hansen, G.N., Williamson, M. & Grimmelikhuijzen, C.J. (2000). Two-color double-labeling in situ hybridization of whole-mount Hydra using RNA probes for five different Hydra neuropeptide preprohormones: evidence for colocalization. *Cell Tissue Res* **301**, 245-253.
- Hengartner, M.O. (2000). The biochemistry of apoptosis. *Nature* **407**, 770-776.
- Hobmayer, B., Rentzsch, F., Kuhn, K., Happel, C.M., von, L., Snyder, P., Rothbacher, U. & Holstein, T.W. (2000). WNT signalling molecules act in axis formation in the diploblastic metazoan Hydra. *Nature* **407**, 186-189.
- Hoepfner, D.J., Hengartner, M.O. & Schnabel, R. (2001). Engulfment genes cooperate with ced-3 to promote cell death in *Caenorhabditis elegans*. *Nature* **412**, 202-206.
- Hoffmeister, S.A. & Schaller, H.C. (1985). A new biochemical marker for foot-specific cell differentiation in hydra. *Roux's Arch.Dev.Biol.* **194**, 453-461.

- Holmes, W. (1950). The mesoglea and muscle fibers of *Clorohydra viridissima*. *Q.J.Microscop.Sci.* **91**, 419-428.
- Holstein, T. (1981). The morphogenesis of nematocytes in *Hydra* and *Forskalia*: an ultrastructural study. *J Ultrastruct Res* **75**, 276-290.
- Holstein, T. (1995). Cnidaria: Hydrozoa / Kamptozoa. In *Süsswasserfauna von Mitteleuropa* (Schwoerbel, J. and Zwick, P., eds.), Gustav Fischer Verlag, Stuttgart;Jena;New York; pp. 1-110.
- Holstein, T.W., Hobmayer, E. & David, C.N. (1991). Pattern of epithelial cell cycling in hydra. *Dev Biol* **148**, 602-611.
- Hughes, A.L. & Yeager, M. (1998). Natural selection at major histocompatibility complex loci of vertebrates. *Annu Rev Genet* **32**, 415-435.
- Humphreys, T. & Reinherz, E.L. (1994). Invertebrate immune recognition, natural immunity and the evolution of positive selection. *Immunol Today* **15**, 316-320.
- Jacobson, M.D., Weil, M. & Raff, M.C. (1997). Programmed cell death in animal development. *Cell* **88**, 347-354.
- Kanaev, I.I. (1969). *Hydra. Essays on the Biology of Fresh Water Polyps*. pp. 1-452.
- Kerr, J.F., Wyllie, A.H. & Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-257.
- Kishimoto, Y., Murate, M. & Sugiyama, T. (1996). Hydra regeneration from recombined ectodermal and endodermal tissue. I. Epibolic ectodermal spreading is driven by cell intercalation. *J Cell Sci* **109 (Pt 4)**, 763-772.
- Kitanaka, C. & Kuchino, Y. (1999). Caspase-independent programmed cell death with necrotic morphology. *Cell Death Differ* **6**, 508-515.
- Kolenkine, X. (1958). Evolution des hydres chimères obtenues apres hétéro-greffe entre *Hydra attenuata* et *Pelmatohydra oligactis*. *C.R.Acad.Sci.* **246**, 1748
- Korthout, H.A., Berecki, G., Bruin, W., van, D. & Wang, M. (2000). The presence and subcellular localization of caspase 3-like proteinases in plant cells. *FEBS Lett* **475**, 139-144.
- Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T.J., Kirschner, M.W., Kohts, K., Kwiatkowski, D.J. & Williams, L.T. (1997). Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* **278**, 294-298.
- Krammer, P.H. (2000). CD95's deadly mission in the immune system. *Nature* **407**, 789-795.

- Lee, H.-T. & Campbell, R.D. (1979). Development and behavior of an intergeneric chimera of Hydra (Pelmatohydra oligactis interstitial cells: Hydra attenuata epithelial cells). *Biol Bull* **157**, 288-296.
- Lenhoff, H.M. & Brown, R.D. (1970). Mass culture of hydra: an improved method and its application to other aquatic invertebrates. *Lab Anim* **4**, 139-154.
- Littlefield, C.L., Finkemeier, C. & Bode, H.R. (1991). Spermatogenesis in Hydra oligactis. II. How temperature controls the reciprocity of sexual and asexual reproduction. *Dev Biol* **146**, 292-300.
- Lockshin, R.A. & Williams, C.M. (1964). Programmed cell death. II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkworms. *J. Insect Physiol.* **10**, 643
- Lohmann, J.U. & Bosch, T.C. (2000). The novel peptide HEADY specifies apical fate in a simple radially symmetric metazoan. *Genes Dev* **14**, 2771-2777.
- Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A. & Jacks, T. (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362**, 847-849.
- Lubbock, R. (1980). Clone-specific cellular recognition in a sea anemone. *Proc Natl Acad Sci U S A* **77**, 6667-6669.
- Lyon, M.K., Kass, S. & Hufnagel, L.A. (1982). Ultrastructural analysis of nematocyte removal in Hydra. *Tissue Cell* **14**, 415-424.
- Meier, P., Finch, A. & Evan, G. (2000). Apoptosis in development. *Nature* **407**, 796-801.
- Meinhardt, H. & Gierer, A. (2000). Pattern formation by local self-activation and lateral inhibition. *Bioessays* **22**, 753-760.
- Meredith, J.E., Fazeli, B. & Schwartz, M.A. (1993). The extracellular matrix as a cell survival factor. *Mol Biol Cell* **4**, 953-961.
- Mokady, O. & Buss, L.W. (1996). Transmission genetics of allorecognition in *Hydractinia symbiolongicarpus* (Cnidaria:Hydrozoa). *Genetics* **143**, 823-827.
- Montero, J.A., Ganan, Y., Macias, D., Rodriguez, L., Sanz, E., Merino, R., Chimal, M., Nieto, M.A. & Hurle, J.M. (2001). Role of FGFs in the control of programmed cell death during limb development. *Development* **128**, 2075-2084.
- Mueller, J.F. (1950). Some observations on the structure of hydra, with particular reference to the muscular system. *Trans. Amer. Microscop. Soc.* **69**, 133-147.
- Munck, A.C. & David, C.N. (1985). Cell proliferation and differentiation kinetics during spermatogenesis in *Hydra carnea*. *Roux. Arch. Dev. Biol.* **194**, 247-256.
- Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S. & Dixit, V.M. (1998). An induced proximity model for caspase-8 activation. *J Biol Chem* **273**, 2926-2930.

- Nagata, S. (2000). Apoptotic DNA fragmentation. *Exp Cell Res* **256**, 12-18.
- Odoorio, T., Rodriguez, T.A., Evans, E.P., Clarke, A.R. & Burgoyne, P.S. (1998). The meiotic checkpoint monitoring synapsis eliminates spermatocytes via p53-independent apoptosis. *Nat Genet* **18**, 257-261.
- Olie, R.A., Durrieu, F., Cornillon, S., Loughran, G., Gross, J., Earnshaw, W.C. & Golstein, P. (1998). Apparent caspase independence of programmed cell death in Dictyostelium. *Curr Biol* **8**, 955-958.
- Percell, K., Willner, J., Signorovizch, T., Holstein, T., Campbell, R. D., and Martinez, D. (2001) A molecular phylogeny of the genus Hydra. In: *International workshop on Hydra and evolution of metazoan development*. (David, C. N. and Bosch, T. C. org.) Evangelische Akademie Tutzing, Germany. p.58.
- Platt, N., da, S. & Gordon, S. (1998). Recognizing death: the phagocytosis of apoptotic cells. *Trends Cell Biol* **8**, 365-372.
- Print, C.G. & Loveland, K.L. (2000). Germ cell suicide: new insights into apoptosis during spermatogenesis. *Bioessays* **22**, 423-430.
- Raff, M.C. (1992). Social controls on cell survival and cell death. *Nature* **356**, 397-400.
- Rao, L., Perez, D. & White, E. (1996). Lamin proteolysis facilitates nuclear events during apoptosis. *J Cell Biol* **135**, 1441-1455.
- Reddien, P.W., Cameron, S. & Horvitz, H.R. (2001). Phagocytosis promotes programmed cell death in *C. elegans*. *Nature* **412**, 198-202.
- Reynolds, A.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208-212.
- Rich, T., Allen, R.L. & Wyllie, A.H. (2000). Defying death after DNA damage. *Nature* **407**, 777-783.
- Rinkevich, B. (1995). Immune responsiveness in marine invertebrates revisited: the concourse of puzzles. In *New Directions in Invertebrate Immunology* (Soderhall, K., Soderhull, K. and Iwanaga, S., eds.), pp. 55-90.
- Robertson, A.M.G. & Thomson, J.N. (1982). Morphology of programmed cell death in the ventral nerve cord of *C. elegans* larvae. *J. Embryol. Exp. Morphol.* **67**, 89-100.
- Rodriguez, I., Ody, C., Araki, K., Garcia, I. & Vassalli, P. (1997). An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO J* **16**, 2262-2270.
- Rubin, D.I. & Bode, H.R. (1982). The aberrant, a morphological mutant of Hydra attenuata, has altered inhibition properties. *Dev Biol* **89**, 316-331.

- Rudel, T. & Bokoch, G.M. (1997). Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. *Science* **276**, 1571-1574.
- Ruoslahti, E. (1996). RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol* **12**, 697-715.
- Sachs, L.M., Abdallah, B., Hassan, A., Levi, G., De, L., Reed, J.C. & Demeneix, B.A. (1997). Apoptosis in *Xenopus* tadpole tail muscles involves Bax-dependent pathways. *FASEB J* **11**, 801-808.
- Samoilovich, M.P., Kuznetsov, S.G., Pavlova, M.S. & Klimovich, V.B. (2001). [Monoclonal antibodies against antigens of *Hydra vulgaris* and *Hydra oligactis*]. *J Evol Biochem Physiol* **37**, 262-269.
- Sanger, F., Nicklen, S. & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463-5467.
- Sarras, M.P. & Deutzmann, R. (2001). *Hydra* and Niccolo Paganini (1782-1840)--two peas in a pod? The molecular basis of extracellular matrix structure in the invertebrate, *Hydra*. *Bioessays* **23**, 716-724.
- Sarras, M.P., Madden, M.E., Zhang, X.M., Gunwar, S., Huff, J.K. & Hudson, B.G. (1991). Extracellular matrix (mesoglea) of *Hydra vulgaris*. I. Isolation and characterization. *Dev Biol* **148**, 481-494.
- Sarras, M.P., Yan, L., Grens, A., Zhang, X., Agbas, A., Huff, J.K., St, J. & Abrahamson, D.R. (1994). Cloning and biological function of laminin in *Hydra vulgaris*. *Dev Biol* **164**, 312-324.
- Sarras, M.P., Zhang, X., Huff, J.K., Accavitti, M.A., St, J. & Abrahamson, D.R. (1993). Extracellular matrix (mesoglea) of *Hydra vulgaris* III. Formation and function during morphogenesis of hydra cell aggregates. *Dev Biol* **157**, 383-398.
- Sato, M., Uchida, M., Graner, F. & Tashiro, H. (1994). Quantitative evaluation of tissue-specific cell adhesion at the level of a single cell pair. *Dev Biol* **162**, 77-84.
- Savill, J. & Fadok, V. (2000). Corpse clearance defines the meaning of cell death. *Nature* **407**, 784-788.
- Savill, J.S., Wyllie, A.H., Henson, J.E., Walport, M.J., Henson, P.M. & Haslett, C. (1989). Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* **83**, 865-875.
- Schmid, V., Ono, S.I. & Reber, M. (1999). Cell-substrate interactions in cnidaria. *Microsc Res Tech* **44**, 254-268.
- Schmidt, T. & David, C.N. (1986). Gland cells in *Hydra*: cell cycle kinetics and development. *J Cell Sci* **85**, 197-215.

- Schneider, K.C. (1890). Histologie von *Hydra fusca* mit besonderer Berücksichtigung des Nervensystem der Hydroidpolypen. *Arch.mikrosk.Anat.* **35**, 321-379.
- Schwarz, G. & Wittekind, D. (1982). Selected aminoacridines as fluorescent probes in cytochemistry in general and in the detection of cancer cells in particular. *Anal Quant Cytol* **4**, 44-54.
- Scofield, V.L., Schlumpberger, J.M., West, L.A. & Weissman, I.L. (1982). Protochordate allorecognition is controlled by a MHC-like gene system. *Nature* **295**, 499-502.
- Steinberg, M.S. (1970). Does differential adhesion govern self-assembly processes in histogenesis? Equilibrium configurations and the emergence of a hierarchy among populations of embryonic cells. *J Exp Zool* **173**, 395-433.
- Stidwill, R.P. & Christen, M. (1998). Alteration of fibronectin affinity during differentiation modulates the in vitro migration velocities of Hydra nematocytes. *Cell Motil Cytoskeleton* **41**, 68-73.
- Stoner, D.S. & Weissman, I.L. (1996). Somatic and germ cell parasitism in a colonial ascidian: possible role for a highly polymorphic allorecognition system. *Proc Natl Acad Sci U S A* **93**, 15254-15259.
- Sugiyama, T. & Fujisawa, T. (1978). Genetic analysis of developmental mechanisms in hydra. V. Cell lineage and development of chimera hydra. *J Cell Sci* **32**, 215-232.
- Takahashi, T., Koizumi, O., Ariura, Y., Romanovitch, A., Bosch, T.C., Kobayakawa, Y., Mohri, S., Bode, H.R., Yum, S., Hatta, M. & Fujisawa, T. (2000). A novel neuropeptide, Hym-355, positively regulates neuron differentiation in Hydra. *Development* **127**, 997-1005.
- Takahata, N., Satta, Y. & Klein, J. (1992). Polymorphism and balancing selection at major histocompatibility complex loci. *Genetics* **130**, 925-938.
- Tardent, P. (1974). Gametogenesis in the genus hydra. *Amer.Zool.* **14**, 447-456.
- Tardent, P. (1978). Coelenterata, Cnidaria. In *Morphogenese der Tiere. Handbuch der ontogenetischen Morphologie und Physiologie in Einzeldarstellungen* (Seidel, F., ed.), Fischer Verlag, Stuttgart, pp. 73-415.
- Tardent, P. (1985). The differentiation of germ cells in *Cnidaria*. In *The Origin and Evolution of Sex* (Anonymous Alan R. Liss, Inc., pp. 163-197.
- Technau, U. & Bode, H.R. (1999). HyBra1, a Brachyury homologue, acts during head formation in Hydra. *Development* **126**, 999-1010.
- Technau, U. & Holstein, T.W. (1992). Cell sorting during the regeneration of Hydra from reaggregated cells. *Dev Biol* **151**, 117-127.
- Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence

- weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680.
- Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia, C., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T. & Nicholson, D.W. (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem* **272**, 17907-17911.
- Townes, P.L. & Holtfreter, J. (1955). Directed movements and selective adhesion of embryonic amphibian cells. *J Exp Zool* **128**, 53-120.
- Trembley, A. (1744). *Mémoire pour servir à l'histoire d'un genre de polypes d'eau douce à bras en forme de cornes*. Verbeck Leyden, pp. 1-324.
- Uren, A.G., Rourke, K., Aravind, L.A., Pisabarro, M.T., Seshagiri, S., Koonin, E.V. & Dixit, V.M. (2000). Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol Cell* **6**, 961-967.
- Vaux, D.L., Weissman, I.L. & Kim, S.K. (1992). Prevention of programmed cell death in *Caenorhabditis elegans* by human bcl-2. *Science* **258**, 1955-1957.
- Vogt, C. (1842). *Untersuchungen über die Entwicklungsgeschichte der Geburtshelferkroete (Alytes obstetricans)*. Solothurn
- Walsh, P.S., Metzger, D.A. & Higuchi, R. (1991). Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* **10**, 506-513.
- Wanek, N. & Campbell, R.D. (1982). Roles of ectodermal and endodermal epithelial cells in Hydra morphogenesis: construction of chimeric strains. *J Exp Zool* **221**, 37-47.
- Weil, M., Jacobson, M.D., Coles, H.S.R., Davies, T.J., Gardener, R.L., Raff, K.D. & Raff, M.C. (1996). Constitutive expression of the machinery for programmed cell death. *J. Cell Biol.* **133**, 1053-1059.
- Weissman, I.L., Saito, Y. & Rinkevich, B. (1990). Allorecognition histocompatibility in a protochordate species: is the relationship to MHC somatic or structural? *Immunol Rev* **113**, 227-241.
- Westheide, W. & Rieger, R. (1996). *Spezielle Zoologie*. G.Fischer Stuttgart; Jena; New York,
- Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A. & Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* **18**, 6531-6535.
- Wood, R. (1959). Intercellular attachment in the epithelium of hydra as revealed by electron microscopy. *J.biophys.biochem.Cytol.* **6**, 343-352.

- Wyllie, A.H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284**, 555-556.
- Wyllie, A.H., Kerr, J.F. & Currie, A.R. (1980). Cell death: the significance of apoptosis. *Int Rev Cytol* **68**, 251-306.
- Yawata, A., Adachi, M., Okuda, H., Naishiro, Y., Takamura, T., Hareyama, M., Takayama, S., Reed, J.C. & Imai, K. (1998). Prolonged cell survival enhances peritoneal dissemination of gastric cancer cells. *Oncogene* **16**, 2681-2686.
- Yin, Y., Stahl, B.C., DeWolf, W.C. & Morgentaler, A. (1998). p53-mediated germ cell quality control in spermatogenesis. *Dev Biol* **204**, 165-171.
- Zieler, J. (1972). Zur Gametogenese und Befruchtungsbiologie von *Hydra*. *Wilhelm Roux's Arch.* **169**, 239-267.

8. Appendix

8.1. Abbreviations

μM	micromolar
cm	centimeter
DEPC	diethylpyrocarbonate
DMFA	dimethylformamide
dNTP	deoxynucleotidetriphosphate
dUTP	deoxyuridinetriphosphate
EtOH	ethanol
I.D.	inner diameter
min	minutes
O.D.	outer diameter
PEG	polyethyleneglycole
S.D.	standard deviation
sec	seconds
TdT	terminal deoxynucleotidyl transferase
TEMED	N,N,N',N'-tetraethylmethyldiamine
Tris	Trihydroxymethylamonimethan

8.2. Acknowledgements

I want to thank Professor Thomas C. G. Bosch for enabling my work in his laboratory as well as for strict and careful supervising my studies. I would also like to thank him for a great time we spent together am Roten Turm in Jena discussing science and many other things.

I am obliged to Mrs. Elke Gretscher, Dr. Hans-Jürgen Agricola in Jena, and Mrs. Antje Thomas and Dr. Friederike Anton-Erxleben in Kiel for an excellent technical assistance. I am grateful to them very much.

I am thankful to a few students who used to work with me at different periods of my doctoral study: Carina Dressler (Jena), Daniela Weide (Jena), Maria Lyanguzowa (St.-Petersburg, Kiel), Stefan Siebert (Kiel) and Henning Fedders (Kiel).

I was lucky to have a great creative atmosphere both in Jena and Kiel. I am grateful to all members of the both labs: Christian Beetz, Ingrid Endl, Uri Frank, Klaus Gellner, Manfred Gödel, Jan Lohmann, Beate Reinhardt, Vassili Rybakine, Henrik Stotz, Friederike Anton-Erxleben, René Augustin, Matthias Becker, Andreas Fröbius, Gregory Genihovich, Matthias Habetha, Kostya Khalturin, Sandra Pankow, Stefan Thomsen, Birgit Belusa, Maria Frank, Magrit Kruse, Antje Thomas, Jörg Wittlieb. Special thanks are to our secretaries Tine Griephan and Christa Kuzel.

The list would never be complete without mentioning the people whom I am infinitely grateful for keeping my spirits high during all this time: Professor Vladimir Klimovich, Dr. Marina Samoilovich and Natalia Arbusova.

CURRICULUM VITAE

NAME: Sergey Kuznetsov
DATE & PLACE OF BIRTH: 09.11.1972, Dzhambul, Kazakhstan, USSR
NATIONALITY: Russian
COUNTRY OF PERMANENT RESIDENCE: Russian Federation
PRESENT ADDRESS: c/o Prof. Bosch, Zoological Institute, University of Kiel, Am Botanischen Garten 1-9, 24118 Kiel, Germany

EDUCATION:

<u>Dates</u>	<u>Discipline</u>	<u>Degree</u>	<u>Institution</u>
09.1979 – 06.1989	Secondary school	Secondary school diploma	General school No. 22, Naberezhnye Tchelny, Russia
08. 1990 – 06. 1995	Cell Biology, Histology (8 semesters)	Bachelor of Science (BS)	Department of Cytology and Histology, Biological Faculty, St.-Petersburg State University, Russia
08. 1995 – 12. 1997	Cell Biology (5 semesters)	Master of Science (MS)	Department of Cytology and Histology, Biological Faculty, St.-Petersburg State University, Russia
02.1998 – present	Cell Biology (3 semesters)	-----	Zoological Institute, University of Kiel, Germany

PUBLICATIONS:

- Venturelli Caterina R., Sergej Kuznetsov, Luis M. Salgado, Thomas C. G. Bosch (2000) An IQGAP-related gene is activated during tentacle formation in the simple metazoan Hydra. *Dev Gene Evol* **210** (8/9), 458-463
- Khalturin, K., Kuznetsov, S. and Bosch, T.C.G. (2000) PCR Fluorescence Differential Display. *In: PCR Protocols: Methods and applications.* (ed. J. Bartlett) Humana Press, Totowa, New Jersey. (In press)
- Kuznetsov Sergey, Maria Lyanguzowa and Thomas C. G. Bosch (2001) Role of epithelial cells and programmed cell death in Hydra spermatogenesis. *Zoology* **104**: 25-31
- Samoilovich Marina P., Sergey G. Kuznetsov, Marina S. Pavlova and Vladimir B. Klimovich (2001) [Monoclonal antibodies against antigens of *Hydra vulgaris* and *Hydra oligactis*] *Journal of Evolutionary Biochemistry and Physiology* **37**: 262-269

Erklärung:

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Kiel, Dezember 2001