CHAPTER 2

LITERATURE REVIEW

2.1 Hernia in pig

2.1.1 Testes descent

The testes of all domestic animals develop within the abdominal cavity (near the kidneys) of a male embryo (Ashdown and Hafez, 1993). The testicular descent is defined as the process that developing testes from their initial position in the abdomen into the scrotum. The testes lies then outside of the abdomen within the scrotum, which has a purse like structure derived from skin and fascia of the abdominal wall. Each testis is enclosed by a tough capsule consisting mostly of fibrous tissue. During testicular descent the gonad migrates caudally within the abdomen to the deep inguinal ring (Ashdown and Hafez, 1993). The mechanism of testicular descent is a complex interaction of hormonal and anatomical factors (Table 2.1) (Hutson *et al.*, 2004; Hutson *et al*., 2005; Tomiyama *et al.*, 2005). There are many factors believed to be involved such as differential growth rates of pig or testes, androgens, function of the gubernaculum and attachment of the testis to the gubernaculum (Visser and Heyns, 1995; Edwards *et al.*, 2003). The process of testicular descent in pigs has been reported by Wensing (1986). The largely mesenchymal gubernaculum of the pig extends from the caudal pole of the testis toward the inguinal canal and ends in a knoblike expansion between the differentiating internal and external abdominal oblique muscles (Figure 2.1). Prior to testicular descent the gubernaculum is separated into three parts:

1) The gubernaculum proper, consisting of an intra-abdominal part within the serosal fold and an extra-abdominal part suspended by the visceral peritoneal layer of the vaginal process.
2) The vaginal part which receives the termination of the cremaster muscle.
3) The intravaginal part or the caudal end of the gubernaculum.

Table 2.1 Anatomical phases of testicular descent and their hormonal control. (Hutson *et al.*, 2004)

	Transabdominal Phase	Inguinoscrotal Phase
Anatomy	1. Regression of cranial suspensory ligament 2. Enlargement of genitoinguinal ligament (gubernaculum) - Anchors testes near groin as embryo grows	1. Gerbernaculum migrates from external inguinal ring to scrotum 2. Processus vaginalis grows inside gubernaculum 3. Testis descends inside processus vaginalis 4. Processus vaginalis obliterates after descent
Hormonal Control	1. Testosterone triggers suspensory ligament involution 2. INSL3 (+MIS) stimulates swelling	1. Testosterone controls migration indirectly via GFN and release of CGRP (sensory nerve endings) stimulates growth of gubernacular tip by trophism and chemotaxis

2.1.2 Etiology and physiology of hernia

Hernia is a Latin word meaning rupture. It is a general term used to describe a bulge or protrusion of an organ through the structure or muscle that normally contains it. A hernia occurs when an organ or fatty tissue protrudes through a weak point in the muscles, membrane or tissues surrounding it. Most hernias develop as the result of a combination of factors (Welsh, 1994; Kingsnorth and LeBlanc, 2003). The epidemiologic data support the assumption that the penetrance of the hernia phenotype is the result of a complex interaction between environmental factors and multiple genes (Jansen *et al.*, 2004; Wessem *et al.*, 2003). Hernias are named based on the location where they develop. On the other hand, hernias can be qualified by the name of the cavity from which it escapes (e.g. an abdominal hernia), the regional location (e.g. an inguinal hernia) or sometimes the name of the cavity where it goes (e.g. scrotal hernia) (Welsh, 1994). In swine umbilical and inguinal hernias are the most common developmental defects.

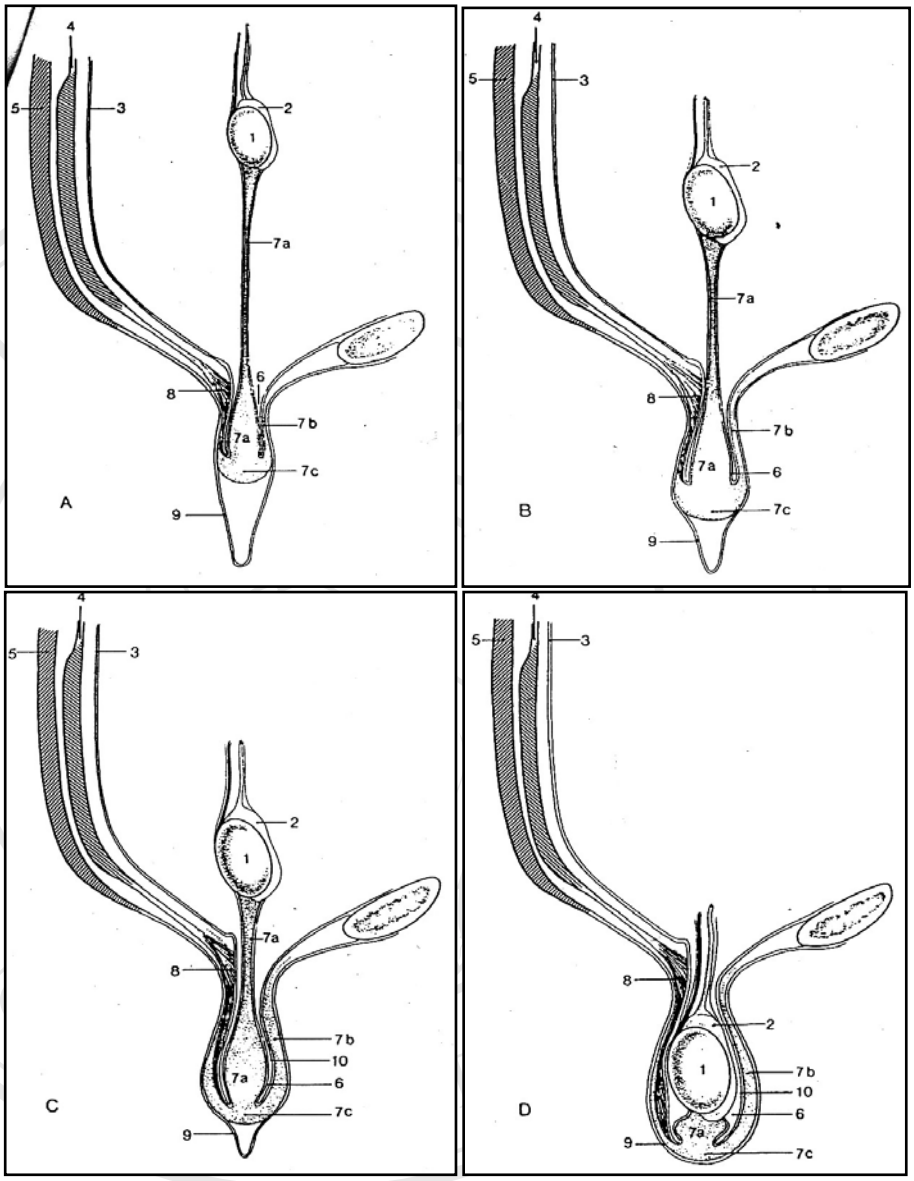

Figure 2.1 Schematic drawing of four phases in the process of testicular descent in the pig fetus: (A) gubernacular relations and development at 65 days, (B) at 75 days, (C) at 85-90 days, (D) at birth; 1. testis; 2. epididymis; 3. parietal peritoneum; 4. internal oblique abdominal muscle; 5. external oblique abdominal muscle; 6. vaginal process; 7a. gubernaculum proper; 7b. vaginal part; 7c. intravaginal part; 8. cremaster muscle; 9. external spermatic fascia; 10. vaginal tunic (parietal layer) (Wensign,1986).

Umbilical hernia

Umbilical hernia appears as a failure of the normal closure of the umbilical ring and results in protrusion of abdominal contents into the overlying sub-cutis. This protrusion is mostly detected when the pigs are between 9 and 14 weeks of age. Umbilical hernia is of less economically importance than inguinal hernia. Its incidence has been estimated with over 1% in females and 0.6% in males (Warwick 1926 cited by Edwards and Mulley, 1999). Occasionally, the hernia becomes so large that surgery or an early slaughter is necessary. Umbilical hernia is also thought to be a polygenically inherited condition (Wrathall, 1975 cited by Edwards and Mulley, 1999). Records of purebred pig boars were used to evaluate breed-of-sire associations. American spotted (n=19) and Duroc boars (n=378) were more likely to develop umbilical hernias than pigs sired by Yorkshire boars (n=1,644) (Search-Bernal *et al*., 1994)

Scrotal or inguinal hernia

Scrotal hernia is a sex-limited condition in males that is believed to result from either an abnormal wide inguinal canal or an incomplete obliterated processus vaginalis. The defects result in protrusion of part of the intestine through the abdominal opening of the inguinal canal and into the scrotum. This is thought to be caused by a weakness of the tunica vaginalis, which allows the abdominal contents to be forced into the inguinal canal by the increased intra-abdominal pressures at or after birth (Edward and Mulley, 1999; Bullard, 1975; Tanyel *et al.*, 2002). The persistence of smooth muscle and myofibroblast have been suggested to define the clinical outcome of a hernia (Tanyel *et al*., 2002). So far, no primary biochemical defect has been reported in pigs. Scrotal hernias occur more frequently on the left side (Todd, 2003) and might not develop until the pig is some weeks or months old (Edwards and Mulley, 1999).

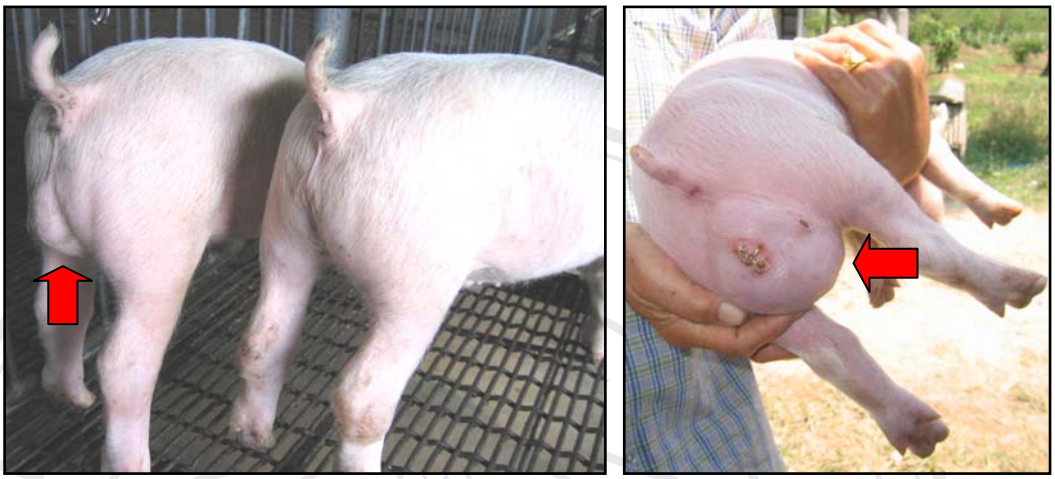

Figure 2.2 Scrotal hernia piglets.

2.2 Genetic background of porcine scrotal hernia

Scrotal hernia in pigs is a congenital disorder whose heritabilities range between 0.2 to 0.6 (Mikami and Fredeen, 1979; Kanp, 1986; Deeb *et al.*, 2004). Warwick (1926) cited by Edwards and Mulley (1999) reported that the frequency of hernia incident will increase from 1.68% to 42% in 2 generations when using a scrotal herniated boar. Thaller *et al*. (1996) also reported a high heritability (0.63-0.70) of scrotal hernia in Dutch Landrace and Pietrain pigs. In Columbia Vogt and Ellersieck (1990) reported the heritability of susceptibility to scrotal hernia based on 5,711 Duroc sires (0.29±0.17), 2,227 Landrace sires (0.34± 0.23) and 2,494 Yorkshire sires (0.34± 0.19) over a 9 year period. They investigated that, the frequencies of scrotal hernia among male full siblings of affected males was consistently higher than the overall frequency of the defect among progeny in each of their respective breed of boar groups. Differences among sires within the Duroc and Landrace boars were significant ($p<0.001$ and $p<0.05$, respectively) but there were no differences in the Yorkshire group.

Back to 1999, a research program at the Institute of Veterinary Medicine (IVM) in Göttingen, Germany was established to decipher the molecular genetics of hernia scrotalis. A genome scan with DNA-markers and affected siblings revealed five chromosomal regions that are associated with the hernia phenotype on porcine chromosome 3, 6, 7, 12 and 15 in German pig breeds (Bornemann-Kolatzki, 2004;

Knorr *et al*., 2001). Moreover, Grindflek *et al*. (2006) reported the QTLs regions of inguinal hernia in Norway pigs were detected in porcine chromosome 1, 2, 5, 6, 15, 17 and SSCX.

Knorr *et al*. (2004) characterized the INSL3 gene encoding leydig cell insulin-like hormone, which plays an important role in male fertility and presumably for congenital disorders of the reproductive tract in pigs. They described the detection of two SNPs in the porcine INSL3 gene and screened a large number of affected pigs (n=223). The polymorphisms in the porcine INSL3 gene showed different frequencies among breeds but no significant association with hernia inguinalis was observed. Thus, they excluded the porcine INSL3 gene as a common genetic basis for scrotal hernia in pig.

Beck *et al*. (2006) reported the analysis of porcine GUSB gene as a candidate gene for congenital hernia inguinalis. The hypothesis of the study is that the swelling of the gubernaculum exceeds through accumulation of hyaluronan due to a diminished degradation. The inguinal ring is thus extended in an unphysiological way and may remain open, predisposing the male pigs for inguinal and scrotal hernia. The limitation of hyaluronan degradation may be based on mutations in the hyaluronan degrading enzymes. GUSB was chosen as functional candidate gene because of its involvement in degradation of hyaluronan within the gubernacular tissue. GUSB also attained status as a positional candidate gene by its localization within a hernia-associated chromosomal region. Five SNPs have been detected in porcine GUSB gene and the association analysis with porcine hernia was carried out. However, no significant associations with hernia inguinalis in the pigs were observed.

In humans, the collagen I/III and MMP-I/-I3 genes have been studied as candidate genes in primary inguinal hernia (Rosch *et al.*, 2002) with the hypothesis that an abnormal collagen metabolism is thought to play an important role in the development of a primary inguinal hernia. They concluded that abnormal changes of type I and type III collagen mRNAs contribute to the development of primary inguinal hernia, whereas the expression of MMP-I and MMP-I3 mRNA appears not to be involved in the development of primary inguinal hernia.

2.3 Scrotal hernia and apoptosis

Lining of scrotum and covering of the testis were used to describe the origin of scrotal or inguinal hernias frequently encountered in pigs. The gubernaculum testis is a connective tissue that connects to the caudal pole of the testis and mediates testicular descent mechanically. The gubernaculum grows rapidly between 60 and 80 days of gestation then the growth rate decreases between 80 and 90 days. The regression of the gubernaculums is distinct after 90 days and progressive until birth. During testicular descent, two major changes in the gubernaculum take place, first an outgrowth and secondly a regression (Wensing, 1986; Fentener Van Vlissingen *et al.*, 1989).

In the porcine fetus enlargement of the gubernaculum is not only due to an increase in its water and glycosaminoglycans content but also to cellular hyperplasia and hypertrophy. The development of muscles in the gubernaculum remains controversial. After descent the gubernaculum regresses and transforms from mucoid to fibrous connective tissue with increasing collagen concentration (Heyns *et al.*, 1990). The gubernaculum testis is of soft, gelatinous consistency and has a club shape during translocation in the fetal bovine (Edwards *et al.*, 2003). There is no appreciable adhesion between the gubernaculum and the surrounding tissues within the inguinal canal and the scrotum at any stage during translocation. When the testes have passed through the inguinal canal, the mesenchyme between the end of the gubernaculum and the body of the scrotum becomes distinctly fluid. Although its role remains controversial, the processus vaginalis is one of the structures proposed to be important in the process of testicular descent. Continued patency of the processus vaginalis, which should normally obliterate after the descent of testes, is accepted to be the principal factor in the development of congenital hernia (Tanyel *et al.*, 2002, Tanyel, 2004b). However, the mechanism of failed obliteration remains unexplained (Tanyel, 2004a). The persistence of smooth muscle hinders the obliteration of the processus vaginalis. Electron microscopy suggested a dedifferentiation of smooth muscle within the sac walls associated with congenital inguinal hernia (Tanyel *et al.*, 2001). Apoptosis is the critical control mechanism in morphogenesis and in normal cell turnover of tissues. Persistence of the smooth muscle has been proposed to result from a failure in apoptosis. Therefore, the smooth muscle around the processus vaginalis

and/or gubernaculums appears to present transiently, whereas apoptosis should take part in its disappearance (Tanyel *et al.*, 2002). The possible pathway of obliteration of the processus vaginalis is shown in Figure 2.3 (Tanyel and Okur, 2004). The initial step appears to be the activation of phospholipase C via G-protein-linked signal transduction. Depletion of Ca^{2+} stores with an increase in cytosolic Ca^{2+} is succeeded by mitochondrial Ca^{2+} overload. Increasing in *BAX* and *Fas* regulated targeting of *BAX* to mitochondria initiate the cascade of PCD. The disappearance of the SM is followed by the disappearance of the mesothelium, thus the obliteration of the PV (Tanyel and Okur, 2004). Since Ca^{2+} is involved in obliteration, different Ca^{2+} contents may reflect the differences in inhibition of PCD among PV associated with inguinal hernia, hypdrocele or undescended testis (Tanyel *et al*., 2003).

2.3.1 Apoptosis

Apoptosis is a normal physiological form of cell death that plays a key role in the maintenance of adult tissues and occurs during the development of all embryonic animals (Jacobson *et al.,* 1997; Cooper, 2000; Meier *et al.,* 2000). Cell death during mammalian embryogenesis starts at the blastula stage and continues throughout the entire life as a mean of keeping tissue homeostasis (Heiser *et al*., 2004). In adults, programmed cell death is responsible for balancing cell proliferation and maintaining constant cell number in tissues turnover or tissue homeostasis and defense against pathogens. Apoptosis is a highly regulated process of cell deletion (Adams and Cory, 1998; Cooper, 2000; Fadeel and Orrenius, 2005). In organisms cells self-destroy when no longer needed in organogenesis or when cells damaged by activating the genetically controlled machinery that lead to apoptosis. Deregulations of apoptosis have been implicated as a fundamental pathogenetic mechanism in a variety of diseases. Loss of muscle cells as a result from enhanced apoptosis has been observed as well in pathological muscle tissue in early postnatal life (Sandri *et al.,* 1999; Zhang *et al*., 2004).

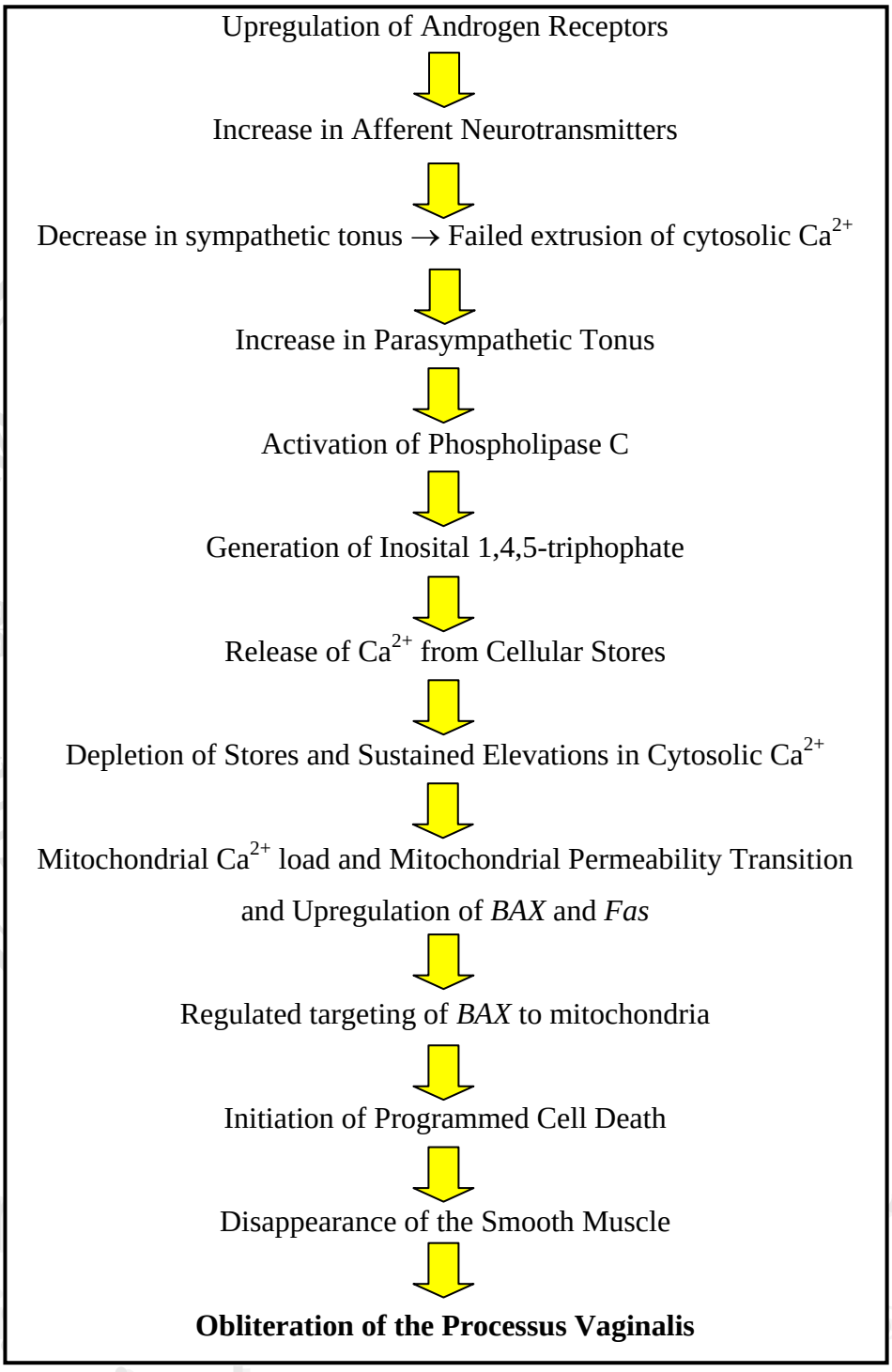

Figure 2.3 Possible pathway of obliteration of the processus vaginalis (Tanyel and Okur, 2004).

Regulation of programmed cell death is mediated by the integrated activity of a variety of signaling pathways (Cooper, 2000) (Figure 2.4). The process of programmed cell death is a proteolytic system that involves a caspases family. Caspases are activated through two main pathways. First, termed the extrinsic pathway, involves ligand binding at the cell surface receptors of the tumor necrosis factor family such as Fas and Fas-ligand system. Activation of this system directly executes programmed cell death in some cells, involvement of mitochondria is essential in other cells. Secondary, involvement of the mitochondria, termed the intrinsic pathway, depends on the depletion of Ca^{2+} stores via G-protein linked signal transduction. Activation of phospholipase C, and thus generation of diacylglycerol and inositol 1, 4, 5-trisphosphate (IP3), is one of the initial steps.

2.3.2 Preprotachykinin A or Tachykinin precursor 1 (*TAC1*) gene

The tachykinins are a family of structurally related neuropeptides and involved in a variety of physiological processes (Liu *et al*., 1999, Severini *et al*., 2005). *TAC1* transcript variant beta encodes the full-length form of this gene. It encodes hormones substance P (SP), neurokinin A and the neuropeptide K (Kang *et al*., 2004). *TAC1* gene is expressed in many regions of the central and peripheral nervous system, as well as in non neuronal tissues (Zimmer *et al*., 1998). The biologic effects of tachykinins have been essentially described on smooth muscle and in the nervous system (Graham *et al.*, 2004). Tachykinin may also be involved in reproduction (Pintado *et al*., 2003)

It has been suggested that substance P is involved in apoptosis. The substance P interacts with the neurokinin-1 receptor (NK1R) to activate members of the mitogen-activated protein kinase (MAPK) cascade, including extracellular signal-regulated kinases 1 and 2 and p38MAPK (Figure 2.5) (Castro-Obregón *et al.*, 2004). Rio *et al*. (2001) tested the SP ability capable of swelling mitochondria and inducing the cleavage of Caspase-3 zymogene in a cell-free system. They investigated that SP was capable of releasing Cytochrome C from mitochondria and activating caspase 3 (Figure 2.6). Although, these pathways are often activated under different conditions they can lead to both growth and apoptosis (DeFea *et al.*, 2000). *TAC1* deficiency also

leads to a decrease in expression of *BAX* and Caspase3 (Liu *et al.*, 1999). These findings indicate that SP could be involved in a seizure-induced cell death pathway involving *BAX* and Caspasses. SP slightly induces p53 and highly induces BCL-2, inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production. This may contribute to prevent the signals of apoptosis by *BAX* via Fas and Caspase-8 activation (Kang *et al.*, 2004). Moreover, Santoni *et al*. (2003) indicated that SP played a protective role in the regulation of thymocyte cell death. Exogenously administered SP completely nullified the apoptotic effect of capsaicin-induced both in the cortex and medulla.

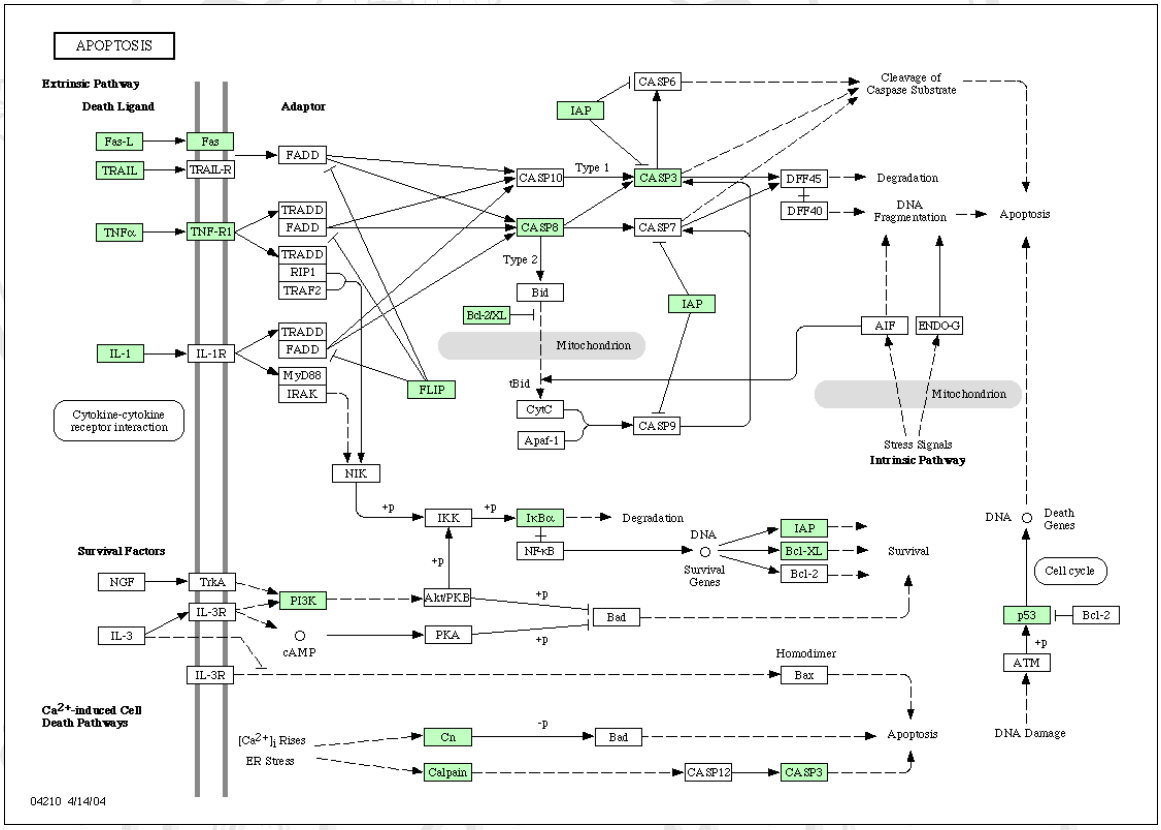

Figure 2.4 Apoptosis pathway (http://www.genome.jp/kegg/pathway/ssc/ssc04210.html).

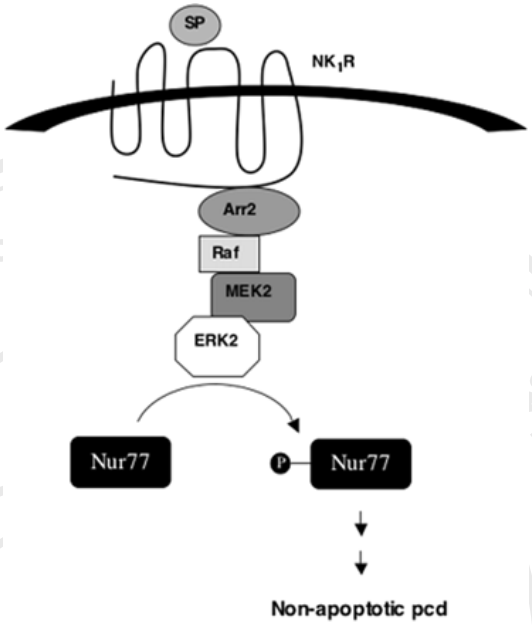

Figure 2.5 Integrative model of the molecular pathway that mediates SP/NK_1R death signaling (Castro-Obregón *et al.*, 2004).

It has been reported that tachykinins may have a modulatory role on the hypothalamo-pituitary axis and the ability to modulate the testicular function by acting directly on testicular cells (Debeljuk *et al.*, 2003). Tamura *et al*. (1997) reported the presence of SP in testicular dorsal root ganglion neurons, in the nerve bundle in the spermatic cord near the testes, and in the tunica vaginalis visceralis of the dog testes. Chiwakata *et al*. (1991) investigated that the SP are potentially paracrine substances regulating intratesticular function. Most interestingly localization of SP was also demonstrated in the fibers associated with the ducts of the rete testis in the pig (Lakomy *et al.*, 1997).

Cunningham *et al*. (2005) identified eight polymorphisms in the *TAC1* by a sequencing approach. They found a SNP in intron 1 which showed significant associations with the multiple sclerosis disease an inflammatory disease of the central nervous system (p=0.009). Mice with disruption of the *TAC1* are resistant to kainate excitoxicity and both necrosis and apoptosis of hippocampal neurons are prevented. Although kainate injections induce expression of the intracellular cell death mediators *BAX* and Caspase3 in wild-type mice, expression is not altered by kainate injection in the mutant mice.

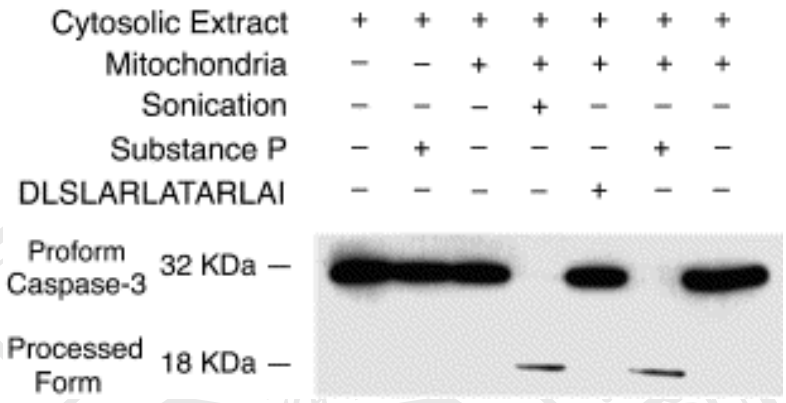

Figure 2.6 Pro-apoptotic activity of SP. The release of cytochrome *c* from mitochondria and the processing of caspase-3 into the active form are shown for SP and controls (sonication, the detergent Triton X-100 and a non-toxic peptide DLSLARLATARLAI) (Rio *et al.*, 2001).

2.3.3 BCL2-associated X protein (*BAX*) Gene

The BCL2-associated X protein is a pro apoptotic member of the BCL-2 protein family that plays a central role in the regulation of program cell death. The BCL-2 protein family with at least 15 members has been identified in mammalian cells and can be divided into three different groups based on BCL-2 homology (BH) domains and function (Figure 2.7) (Joza *et al.*, 2002). The anti-apoptotic members typically have BH1 through BH4 domains such as BCL-2 and BCL-XL. The pro-apoptotic members can be divided into two groups. The first group contains only BH3 domain such as BAD and BIM. The second group contains BH1, BH2 and BH3 domains such as *BAX* and *BAK*. This related protein family plays key roles in the regulation of apoptosis and the individual members can function to either block or promote programmed cell death and also play a pivotal role in deciding whether a cell will live or die (Wolter *et al.*, 1997; Gross *et al.*, 1999).

Seven alternative spliced transcript variants encoding different isoforms have been reported for *BAX* (Table 2.2 and Figure 2.8). *BAX* alpha (*BAX*-α) is encoded by 6 exons that predict a 21 kDa membrane protein. *BAX* beta (*BAX*-β) possessed an unspliced intron 5 and lacks a transmembrane domain. *BAX* gramma (*BAX*-γ) has two

forms, one similar to *BAX* β but missing exon 2, and another lacking exon 2 results in a translational frame shift termination in exon 3. *BAX*-delta (*BAX*-δ) lacks exon 3 but maintains the original reading frame, including the functionally critical C-terminal membrane- anchoring region. *BAX*-omega (*BAX*-ω) has an extra insert between exons 5 and 6, which results in a translational frame shift and loss of the transmembrane domain. *BAX* epsilon (*BAX*-ε) is missing the last 69 amino acids of *BAX*α, encompassing the BH2 and TM domain. *BAX* kappa (*BAX*-κ), is a splice variant of *BAX* from ischemic rat brain with conserved BH1, BH2 and BH3 binding domains and a C-terminal transmembrane domain (TM), but with an extra 446 bp insert between exons 1 and 2 leading to the loss of an N-terminal ART domain (Jin *et al.*, 2001).

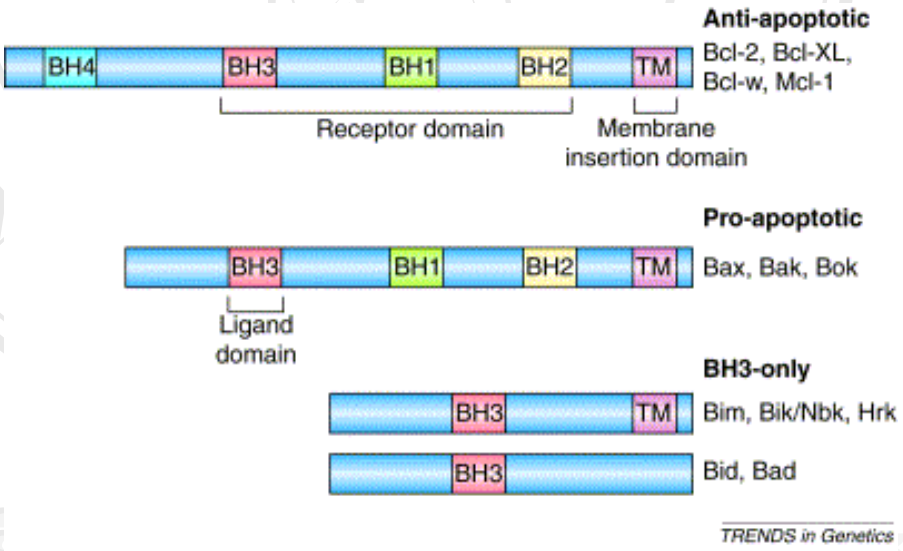

Figure 2.7 Members of the BCL-2 family share variable amounts of conserved BH (BCL-2 homology) sequence motifs (Joza *et al.*, 2002).

Table 2.2 Transcription variants of Human *BAX* gene.

Transcription variants	GenBank accession no	Molecular weight (Protein; kDa)	Number of amino acid
BAX-alpha	NM_138761	21	192
BAX-beta	NM_007324	24	218
BAX-gramma	NM_138762	4.5	41
BAX-delta	NM_138763	16	143
BAX-epsilon	NM_138764	24	221
BAX-kuppa	AF_235993	18	164
BAX-sigma	NM_138765	19	173

The structure of the human *BAX* protein consists of 9 α helices (Figure 2.9). Suzuki *et al*. (2000) suggested that the structure of *BAX* implies that the hydrophobic pocket plays an important role in apoptosis initiation. The side chains of the hydrophobic residues in the BH3 domain of *BAX* proposed to be important for dimmer formation are oriented towards the center hydrophobic core of the protein (Chao and Korsmeyer, 1998). Upon induction of apoptosis *BAX* binds mitochondria apparently via its C-terminal helix as deletion of the last five or more amino acids prevent mitochondrial binding. Therefore, the conformational change that allows the C terminus to enter mitochondrial membranes must disengage helix α9 from the protein core, and thus expose the hydrophobic BH3 binding pocket to participate in dimmer formation.

The human *BAX* gene has been mapped to chromosome 19q13.3-q13.4 (Apte *et al.*, 1995). Mutations in the promoter and coding regions of the human *BAX* gene have been reported. The presence of a G to A substitution at position 125 (G125A) in human *BAX* promoter was associated with lower *BAX* mRNA (P=0.004) and protein (P=0.024) levels in chronic lymphocytic leukemia patients (Moshynska *et al.*, 2003; 2005). However, Starczynski *et al*. (2005) and Skogsberg *et al*. (2006) reported that this polymorphism does not lead to the development of chronic lymphocytic leukemia or affects disease progression.

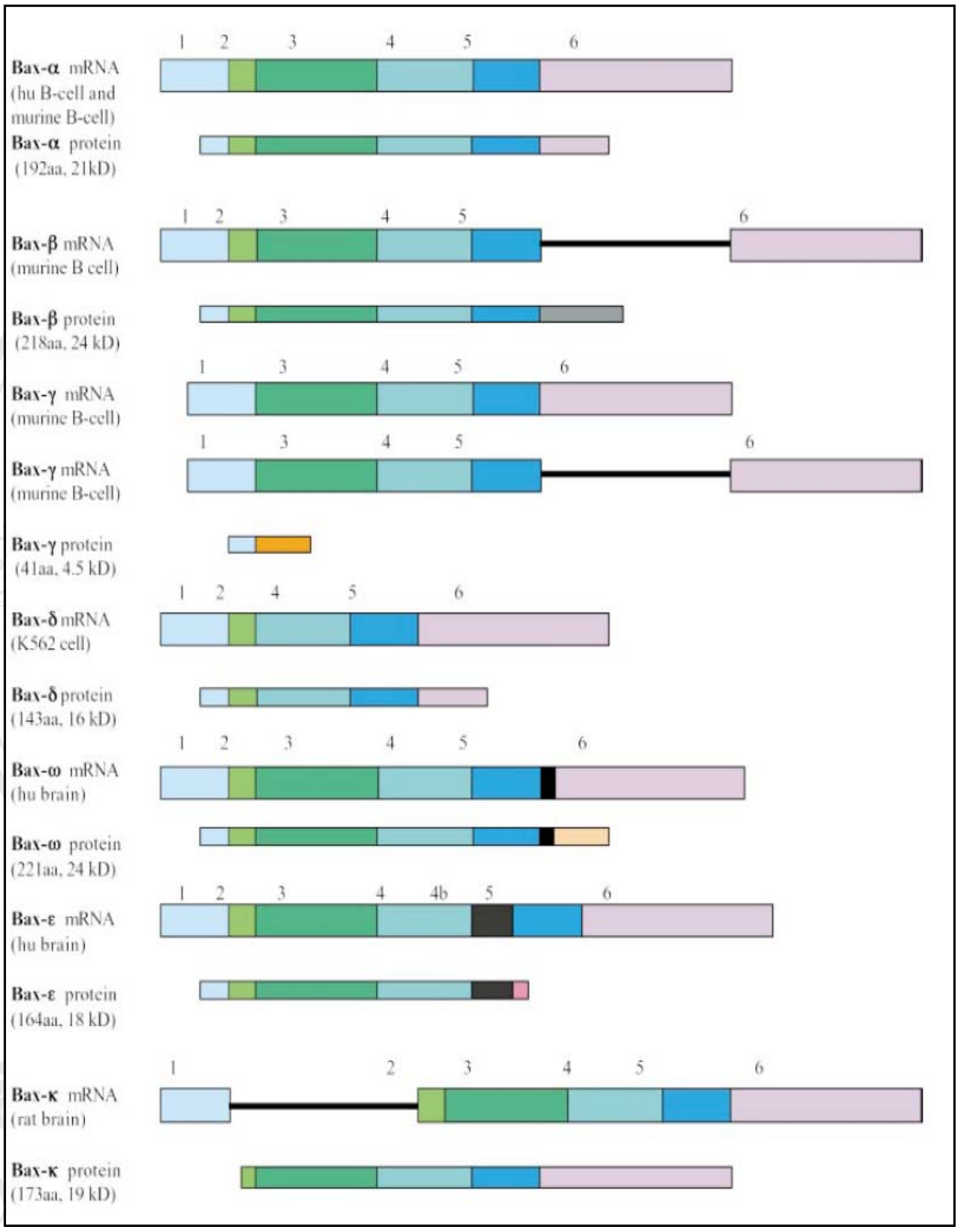

Figure 2.8 Alternative *BAX* transcripts and their predicted protein products. Boxes indicate exons, which are identified by numbers. DNA inserts are indicated by bold line. Hu, human; aa, amino acid (Jin *et al.*, 2001).

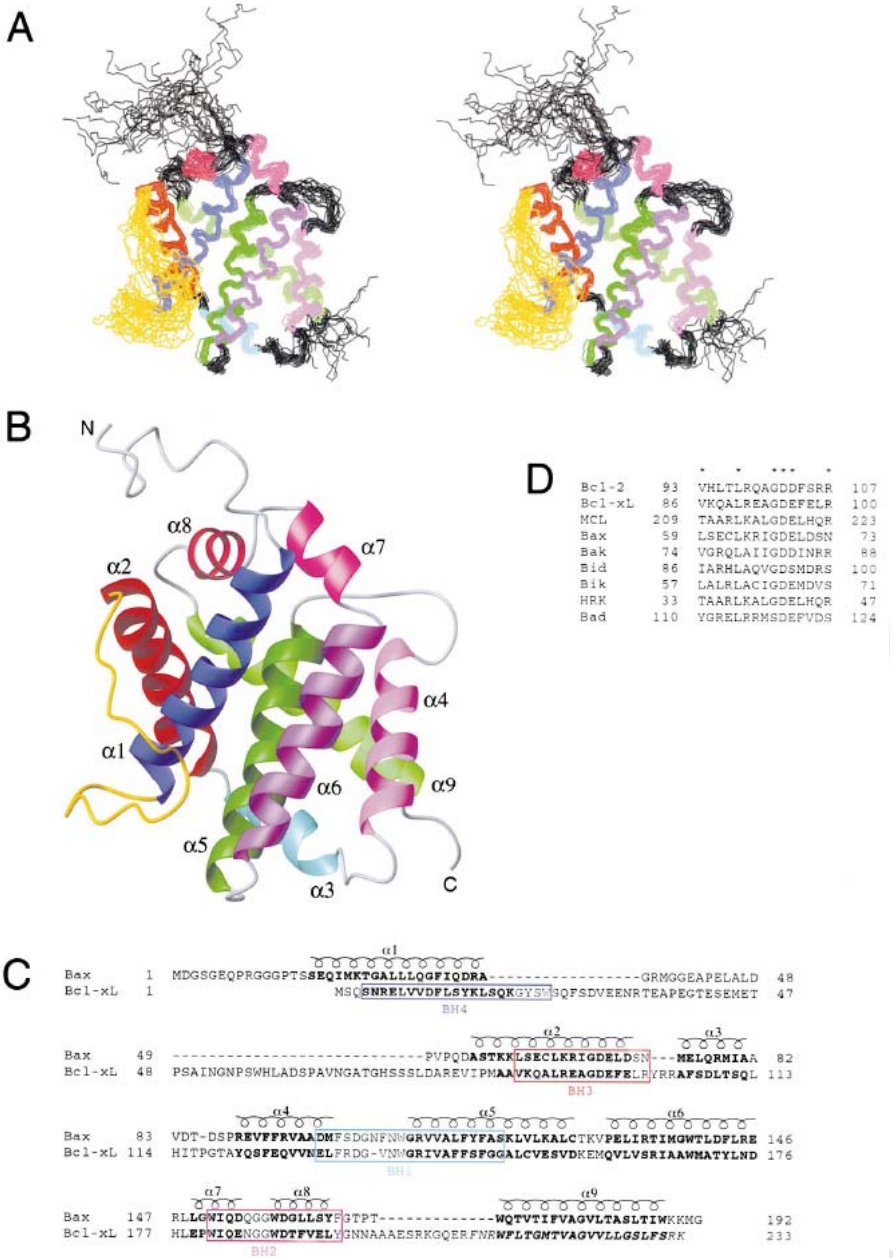

Figure 2.9 Structure of the BAX protein. (A) A stereo view of the backbone. (B) A ribbon representation of an averaged minimized NMR structure for BAX. Helices are distinguished by different colors. (C) Amino acid sequence alignment between BAX and Bcl-xL based on their structures. (D) Multiple sequence alignment of conserved BH3 domain among BCL-2 family proteins. Highly conserved residues are marked by asterisks (Suzuki *et al.*, 2000).