

**Molecular characterization of the porcine hyaluronidase gene
cluster on *SSC13q21***

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To my family...

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1 LIST OF PUBLICATIONS

This thesis is based on the following papers:

A. Peer-reviewed journals:

Gatphayak K., Knorr C., Habermann F., Fries R. and Brenig B. (2003): Assignment of the porcine hyaluronidase-3 (*HYAL3*) gene to *SSC13q21* by FISH and confirmation by hybrid panel analyses. *Cytogenet. Genome Res* **101**, 178F

Gatphayak K., Knorr C. and Brenig B. (2003): Assignment of the porcine *FUS1* gene to *SSC13q21-q22* by somatic cell and radiation hybrid panel mapping. *Cytogenet Genome Res* **103** (in press, DOI:10.1159/000076322)

Gatphayak K., Knorr C., Chen K.F. and Brenig B. (2004): Structural and expression analysis of the porcine *FUS2* gene. (accepted for publication in *Gene*; January 22, 2004)

Gatphayak K., Knorr C. and Brenig B. (2004): Molecular characterization of porcine *hyaluronidase* genes 1, 2, and 3 clustered on *SSC13q21*. (accepted for publication in *Cytogenet Genome Res*; January 21, 2004)

Chen K.F., Knorr C., Moser G., Gatphayak K. and Brenig B. (2004): Molecular characterization of the porcine testis-specific phosphoglycerate kinase 2 (*PGK2*) gene and its association with male fertility. (revised version in preparation)

B. Congress contribution:

Gatphayak K., Knorr C. and Brenig B. (2003): Molecular characterization of the porcine hyaluronidase genes cluster on *SSC13q21*. DGfZ/GfT-Convention, Goettingen 17./18. September 2003, B12

2 INTRODUCTION

Molecular genetics is now providing tools to enhance rates of genetic improvement by selection on quantitative trait loci (QTL) or on linked markers. Therefore, molecular genetics enables to identify genes affecting quantitative traits. The new knowledge applied in pig genomics coupled with global methods of detecting genetic abnormalities will provide implications for clinical medicine.

The aim to study hyaluronidase genes (*HYAL*) is lying in their ability to be candidate genes for cancer, fertilization mechanisms, as well as their role in hyaluronan degradation particularly in the Descensus testis process. Two groups of three genes each were identified on human chromosomes *3p21.3* and *7q31.3*. The genes on chromosome 3 are more closely related to each other and have a similar genomic structure in terms of intron and exon arrangement than the two genes and the pseudogene on chromosome 7 that are not preserved. The porcine *HYAL* genes on chromosome 3 *HYAL1*, *HYAL2* and *HYAL3* were chosen in the beginning of our studies and later *FUS1* and *FUS2* were also included. Although the background of these genes are identified based on being tumor suppressor gene (*TSG*) candidates, (homozygous deletions of the genes of the *3p21.3* region result in tumor suppressor activities), their individual contributions to overall hyaluronan degradation is well documented.

The following molecular genetic studies of the porcine *HYAL* genes report the genes' structure, their chromosomal assignment, their expression patterns and existing polymorphisms. Knowledge of the molecular structure leads to suggest a role for hyaluronidases in biological processes which may be implemented in further studies to use hyaluronidases as a diagnostic marker and the pig as a model animal in cancer research and therapy.

3 HYALURONAN AND HYALURONIDASES

3.1 Structure, physiology and function of hyaluronic acid (hyaluronan)

Hyaluronan or hyaluronic acid (HA) is a member of the family of complex carbohydrates known as glycosaminoglycans (GAGs). It is found in the extracellular matrix especially of soft connective tissues. The chemical structure consists of repeated disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid linked by $\beta(1-4)$ and $\beta(1-3)$ glycosidic bonds (figure 1). Hyaluronan is a large polymer with an average range of approximately 25,000 disaccharide units. Hyaluronan molecules can be stabilized and thereby form a stiffened helical configuration. Water can be trapped within this stiffened conformation (Jacobson, 2002). When the amount of HA in a solution reaches a concentration of approximately 0.1 mg/ml, the molecule forms an entangled network. Inter- and intramolecular interactions occur such that the volume occupied by HA increases, resulting in the exclusion of other macromolecules from their normal molecular environment. This is called steric exclusion that can influence both osmotic activity and transport in the extracellular matrix since water is immobilized and flow-restricted. The overall turnover rate ($t_{1/2}$) is rapid for a connective tissue matrix component with ($t_{1/2} = 0.5$ to a few day) (Laurent and Fraser, 1992). HA is synthesized by HA synthase (HAS) in the plasma membrane and extrudes then into the extracellular space. The synthesis is regulated by various factors in mammals like growth factors, hormones and inflammatory mediators (Jacobson, 2002).

The molecular functions of HA are considered to fall into three partially overlapping categories: First, because of its unique hydrodynamic properties, HA occupies an enormous hydrodynamic domain that influences the hydration and physical properties of the tissues. Second, by its instructive effects on cell signaling and behavior, it interacts with other extracellular matrix macromolecules and hyaluronan-binding macromolecules (hyaladherins) such as versican and aggrecan (Knudson, 1996; Toole, 2001). Finally, HA interacts with cell surface receptors, notably CD44 and RHAMM (receptor for hyaluronan-mediated motility) transducing intracellular signals to regulate cell growth, survival and movement (Turley *et al.*, 2002).

Hyaluronan has also been recognized in clinical medicine as serum hyaluronan is a promising reagent in the diagnosis of liver diseases and various inflammatory conditions (Laurent and Fraser, 1992).

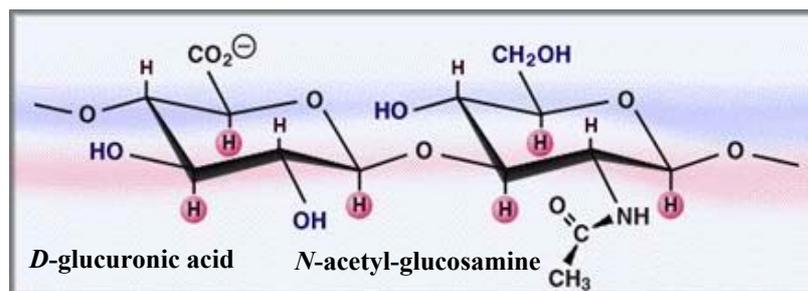


Figure 1: Structure of hyaluronic acid. Hydrogen atoms form a non-polar, relatively hydrophobic face while the equatorial side chains form a more polar, hydrophilic face, thereby creating a twisting ribbon structure (adapted from Laurent and Hascall, 1997).

3.2 Structure, physiology and function of hyaluronidases

In vertebrate tissues, degradation of hyaluronan occurs by the concerted action of three enzymes: a hyaluronidase and two exoglycosidases that remove sugars sequentially from the non-reducing termini, a beta-glucuronidase and a beta-N-acetyl-glucosaminidase. Hyaluronidases (HAases) are enzymes responsible for most of the degradation of HA as they catalyze the random hydrolysis of 1,4-linkages between N-acetyl- β -D-glucosamine and D-glucuronate residues in hyaluronan (Kreil, 1995). The endolytic cleavage by the hyaluronidase generates increasing numbers of non-reducing termini for the exoglycosidases. The relative contribution of the endo- and exocleavage reactions varies from tissue to tissue. Though these enzymes have the same or similar substrates, their mechanisms vary in a wide range of pH optima according to substrate specificity or by their reaction product. Hyaluronidases are members of a class of endoglycosamine enzymes of considerable complexity and heterogeneity.

Three principal types of hyaluronidases have been characterized, mammalian (EC 3.2.1.35), bacterial (EC 4.2.99.1) and hyaluronidases from leeches, other parasites and crustaceans (EC 3.2.1.36) and are grouped as endo-beta-N-acetyl hexosaminidases (mammalian and bacterial) or endo-beta-glucuronidases (leeches, other parasites, and crustaceans) with different end products (Stern and Csoka, 2000). The first eukaryotic hyaluronidase which could be cloned was from the bee venom which is homologous to the PH-20 hyaluronidase found in human spermatozoa (Kreil, 1995; Gmachl and Kreil, 1993).

The testis specific hyaluronidase has long been recognized and characterized. The commercially used hyaluronidase was isolated from sheep and bovine testes, since hyaluronidase plays a vital role in fertilization of the mammalian egg (Baba *et al.*, 2002).

Recently, HAase extracted from Norway lobster (*Nephrops norvegicus*) was also successfully commercially used (Krishnapillai *et al.*, 1999). The examples of applications of hyaluronidases in the pharmaceutical and food industry show that dispersion and absorption of other drug and treatment of cancer (Seravac, 2001) is facilitated considering them as putative virulent factors of pathogenic organisms in cultures (CIRVAL, 2003).

3.2.1 Hyaluronidases in cancer

Interactions between hyaluronan and cells in cancer progression are reviewed by Toole *et al.* (2002). Various tumors are surrounded by a connective tissue matrix (stroma) enriched with hyaluronan. The enzyme hyaluronidase appears to interfere with metastasis, the process by which tumors spread from the primary organ in which they arose to other sites in the body, through three mechanisms. Two of the requirements for cancer cells to metastasize are that they must become motile, and that they must have a space into which to move. Hyaluronan accomplishes both of these tasks. Hyaluronan on the surface of cancer cells hydrates, expands, and opens up spaces around cancer cells, enabling them to invade surrounding tissues. One suggestion is that invasive tumor cells, or products secreted by these cells, induce stimulation of hyaluronan synthesis by stromal cells such as fibroblasts or smooth muscle cells (Knudson and Knudson, 1995). An increase of hyaluronan may provide an embryonic-like environment that facilitates tumor cell migration (Knudson, 1996), as an enhanced of cell motility (migration) is necessary for cells to become metastatic (Zetter Research Lab, 2003). Using animal models, rabbit V2 carcinoma was studied by Toole *et al.* (1979) demonstrating that the accumulation of hyaluronan is correlated with tumor invasiveness.

Hyaluronan also allows tumor cells to protect themselves against immune assaults by such mechanisms as the secretion of non-specific suppressive or anti-inflammatory factors or the shedding of tumor-specific antigens such as lymphocyte-mediated cytotoxicity. Removal of hyaluronan present in the extracellular matrix of tumor cells by hyaluronidase renders the latter more accessible to effector T cells. Experiments showed in transplanted mice suffering two tumors (3LL lung carcinoma and the B16F10 melanoma) that tumor proliferation is influenced by hyaluronidase 1 (HYAL1). Both tumors developed more slowly, measured by a slower rate of increase in local tumor size and by a prolonged survival time, in presence of higher levels of circulating hyaluronidase concentrations (De Maeyer and De Maeyer-Guignard, 1992). Hyaluronidase treatment destroys the hyaluronate coat surrounding tumor cells and allows lymphocytes to approach the tumor

membrane to enhance the cytotoxic action of immune response (McBride and Bard, 1979) and to be more accessible both for tumor-specific cytotoxic-T-cell generation and activity (De Maeyer and De Maeyer-Guignard, 1992).

The hydrodynamic effect of hyaluronan on the extracellular matrix interacts with cell migration, a role mediated via cell surface by hyaluronan-binding sites or hyaluronan receptors. Hyaluronidase breaks down HA and eliminates as a direct effect of this mechanism cancer cell spread. The receptor for HA, the lymphocyte homing receptor CD44, functions as an anchor to bind HA to the cancer cell surface. CD44 comes in a variety of forms, a short form found on normal cells, and longer variants that occur on motile cancer cells only (Knudson, 1996). A portion of CD44 protrudes through the surface membrane into the cell cytoplasm, where it interacts with the cytoskeleton, the machinery for cell movement. Interactions between hyaluronan and CD44 have been reported to play the critical role in the process of tumor metastasis. They promote the formation of tumor embolisms, which in turn, increase the chances that the tumor cells would be trapped in the lungs (Zhang *et al.*, 1995). As shown experimentally, inserting this longer cancer form of CD44 into tumor cells changes them into cancer cells that aggressively metastasize. Hyaluronidase eliminates the cancer cells' ability to move, by converting the long cancer form of CD44 to the short "normal" immobile form. A molecular and cell biology study to determine precisely how hyaluronidase regulates this alternative splicing of CD44 is done in the California Breast cancer research project. Understanding of these events in the regulation of CD44 will finally make it possible to develop a new generation of anticancer drugs to prevent metastases associated with breast cancer progression.

Another receptor RHAMM (an inherent hyalderin), functions primary in stimulating a locomotory, chemokinetic response of tumor cells to soluble hyaluronan. RHAMM does not appear to be directly related to CD44 or hyaluronan-binding proteoglycans (i.e. does not contain B-loop structure) but may share common structural motifs within the actual hyaluronan-binding domain (Knudson, 1996). The ability of intracellular RHAMM forms to associate with multiple signaling complexes and cytoskeleton suggest that they function as adapter proteins like vinculin and paxillin. Hyaluronan-RHAMM interactions induce transient phosphorylation of focal adhesion kinase, p125^{FAK}, leading to initiation of locomotion. Suppression of this interaction inhibits both cell locomotion and proliferation and leads to inhibition of tumor growth. Whereas,

over-expression of RHAMM leads to enhanced tumor growth and metastasis (Toole *et al.*, 2002).

The third function of hyaluronidase was reviewed by Stern (1995). The enzyme causes established breast cancers to shrink. Inoculation of hyaluronidase in mice suffering human breast cancer shrinks the tumor to half its size in four days. The malignant cells cannot maintain motility and thus invasiveness, and the tumor cannot progress or grow and must completely apparently undergo regression. These biological mechanisms are still not understood, but may be a consequence of the two functions as mentioned before. Hyaluronidase may thus prevent progression of human breast cancer, and it seems not to have the devastating toxic side effects of other anticancer agents.

Another role of hyaluronidase in the context of cancer may be the inhibition of tumor cell growth by an increased tumor necrosis factor (TNF) cytotoxicity. HAase counteracts transforming growth factor beta mediated (TGF-beta-mediated) TNF-resistance and suppresses TGF-beta1 gene expression. Hyaluronidase antagonizes TGF-beta-mediated inhibition of epithelial cell growth. Both TGF-beta and hyaluronidase are essential for the progression and invasiveness of breast, prostate and other cancers. A stage-dependent expression, as well as a balanced production of these proteins is essential for cancer development and self protection against TNF cytotoxicity (Chang, 1998). It has been shown that hyaluronidase is able to disrupt intercellular adhesion and to chemosensitize tumor cells by a mechanism, independent of increased drug penetration in cancer chemotherapy (Buschauer, 2003).

3.2.2 Hyaluronidases in fertilization

The process of fertilization begins when the sperm contacts the outermost egg investment (cumulus cells) and ends with fusion of two haploid pronuclei in the egg cytoplasm. Many steps in fertilization involve carbohydrate-based molecular recognition between sperm and egg. Vertebrate egg coats contain zona pellucida (ZP) family glycoproteins whose carbohydrates bind to sperm receptors. Fertilization is a multistep process: sperm must locate and fuse with the egg and the egg must prevent further sperm fusion to avoid pathological polyspermy. An extracellular matrix surrounds eggs. When sperm contacts this matrix, there are primary binding events that lead to the sperm acrosome reaction (AR). The AR is triggered by increases in intracellular Ca^{+} and pH resulting in exocytosis of the acrosomal vesicle in the sperm head. Then the membrane exposed by AR fuses with the egg plasma membrane. Protein-carbohydrate interactions

play a critical role in this complex process (Kathryn and Victor, 2001; Naz and Vanek, 1998).

The protein of the sperm hyaluronidase *SPAMI* (sperm adhesion molecule), PH-20 aids in penetrating the layer of cumulus cells by digesting hyaluronic acid (Gmachl and Kreil, 1993). PH-20 is expressed in testis in two forms. During fertilization, one form is present on the surface of the acrosomal head of sperm which is tethered to the plasma membrane by the lipid anchor glycosylphosphatidylinositol (GPI-anchored). This enables the sperm to penetrate the hyaluronan-rich extracellular matrix of the cumulus cells. The second form is expressed and located in the acrosomal vesicle, a soluble form, which clears the hyaluronan in the ZP rapidly and thereby helps the sperm to reach the oocyte membrane (Brinck, 2000; Stern and Csoka, 2000). Sperm hyaluronidase activity relates significantly to the fertilization rate. The ability of sperm to penetrate a highly viscous solution of sodium hyaluronan is correlated with the motility and fertilization deficiency. Hyaluronan penetration is used as a method to prepare and evaluate the functional competence of human sperm in assisted fertilization programs (Salustri and Fulop, 1998). Two hyaluronidases genes, *HYALPI* and *SPAMI*, are both expressed exclusively in testicular tissues, *HYAL3* is highly expressed in testis but also in bone marrow (Csoka *et al.*, 1999). These two tissues retain a stem cell-like state for life in the animal but whether or not *HYAL3* participates in the process of fertilization is still not known and needs to be elucidated (Stern and Csoka, 2000). A possible participation in fertilization is also not known for *HYAL5* an additional hyaluronidase localized almost 100-kb away from the *SPAMI* gene (Baba *et al.*, 2002).

3.2.3 Hyaluronidases in *Descensus testis*

Two major forms of a misleded Descensus testis are known in mammals: Cryptorchidism and hernia.

Cryptorchidism

During testicular development, the testes descend into the scrotum for a cooler environment to preserve normal spermatogenesis. Cryptorchidism is defined as an absence of at least one testis from the scrotum. Although the other word often used for this interchangeably phenomenon is undescended testis, the terms are not synonymous since cryptorchid testes can also be retractile, ectopic, or absent. Thus, there are 4 types of cryptorchidism: undescended along the path of descent, ectopic testes outside the path of

descent, retractile testes descended but ascend due to the cremaster reflex (genitofemoral nerve) and absent testes vanished testes (intrauterine testicular torsion) or agenesis distinguished by the presence of Mullerian structure. The molecular aetiology of this syndrome was for a long time unclear. Recently, the role of insulin-like factor-3 (INSL3; alternatively called relaxin-like factor) has been highlighted through the cryptorchid phenotype of mice (Nef and Parada 1999; Zimmermann *et al.* 1999). In human and in pig several different mutations in the INSL3 gene have been investigated and association studies to the phenotype cryptorchidism undertaken. However, most of these studies exclude that mutations in the INSL3 are the genetic cause for cryptorchidism (e.g. Takahashi *et al.*, 2001; Baker *et al.*, 2002; Knorr *et al.*, 2003), whereas some report a low association (Tomboc *et al.*, 2000). Studies on developmental transcription factors, such as Hoxa-10 in mice, suggest that other specific molecular cascades could also lead to a cryptorchid phenotype (Ivell and Hartung, 2003).

The inheritance of cryptorchidism was investigated in Duroc swine. Two hypotheses were examined by Rothschild *et al.* (1988): first, that this trait is controlled by homozygosity of a recessive gene at a single locus; second, that it is controlled by homozygosity of recessive genes at two loci. The first hypothesis of a single recessive gene was rejected, but not the two-locus model.

Hernia

With respect to the reproductive tract two forms of hernia are described: hernia inguinalis and hernia scrotalis: Hernia scrotalis (inguinalis) is a sex-limited (not sex-limited) condition in males (both sexes) that is believed to result from a weakness of the musculature surrounding the inguinal canal, permitting the intestine to drop into the scrotum (inguinal canal). Hernias occur much more frequently on the left side of an individual. The mode of inheritance shows familiar occurrence, but convincing evidence for a single gene inheritance is lacking (Rothschild and Ruvinsky, 1998). Several genes have been suggested to be causative for the disorder along with maternal and environmental influences (Todd See, 2003). A study showed recently that the frequency of inguinal hernia could be increased from 1.7% to 90 % in only 6 generations (Wolfe, 2002).

Gubernaculum hyaluronidase and Testicular descent

The gubernaculum testis is the connection tissue organ that is connected to the caudal pole of the testis and which mediates testicular descent mechanically. During the first phase of descent the gubernaculum grows through the inguinal canal into the future cavity and simultaneously brings the testis to the inguinal canal. During the second phase of descent the gubernaculum condenses and thus promotes the further descent of testis into the scrotum (Wensing *et al.*,1988). The mechanisms of testicular descent are displayed schematically in Figure 2. To find biological markers for differentiated expression of gubernaculum fibroblasts characteristic for outgrowth or regression of the gubernaculum testis the composition of the tissue with respect to glycosaminoglycans, collagen and DNA was investigated by Fentener van Vlissingen *et al.* (1989). It became evident that the gubernaculum development with respect to cell proliferation rate, extracellular matrix (ECM) synthesis and degradation is very different during gubernaculum development. The metabolism of hyaluronic acid changed remarkably during testis development. During the first phase of testicular descent (70-80 days of pig fetus), HA synthesis rate exceeds the rate of cell proliferation and concomitant synthesis of sulphated glycosaminoglycans (S-GAG), HA, and collagen. In this phase, the development of HA accumulation exceeds the increase in cell number. Cell density is low because of the increase in hydrated space. Regulatory substances that may control testicular descent should control the first phase of gubernaculum development. These factors must exert their effects on the fibroblasts of the gubernaculum testis and influence characteristics of connective tissue growth and differentiation both with respect to cell proliferation and composition of the ECM. The amount of HA diminishes during the second phase, cell density and S-GAG increases, the total amount of HA per gubernaculum decreases dramatically while the concentration of HA only slightly decreases. That might cause the hydrated space to decrease proportionally to the decrease in HA. Concentration of HA per gram of tissue was stable between 70 days of gestation but after 90 days HA decreased steadily, indicating biodegradation. The ratio between HA and DNA increases between 70 and 90 days of gestation and decreases thereafter. The enzymes that act in the biodegradation of HA might feature the second phase of testicular descent. Characteristic for the hyaluronidase in the gubernaculum are the extremely low pH optimum (2.5-3.5). The enzyme activity per gram of the tissue increases slowly but steadily during the fetal period and at a higher rate postnatally (figure 3a, b). The increase of hyaluronidase between 80 day post conceptionem and date of birth

was due to the increase of cell density, while the postnatal increase represented increased enzyme activity per cell. The selective degradation of HA was probably due to the net increased lysosomal digestion by hyaluronidase and other enzymes. It acts as a distinct feature of gubernaculum development during the second phase. Biodegradation of HA might therefore be useful as a biological marker of gubernaculum regression.

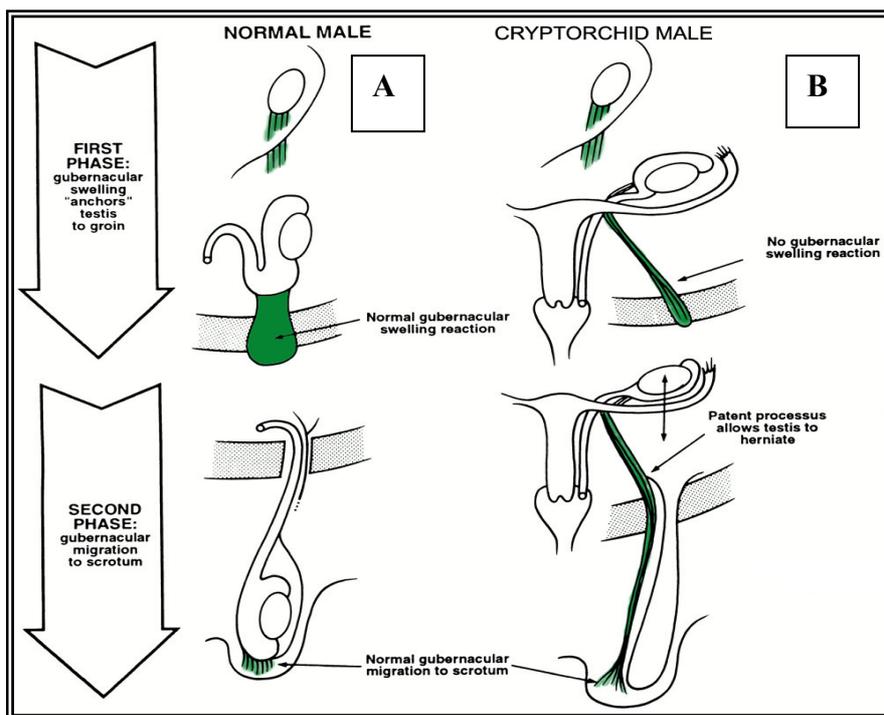


Figure 2: Schema of gonadal descent showing two phases in normal male (A) compared with cryptorchid male (B). Normal development of the processus vaginalis, which is part of the second phase of descent, along with hypermobility of the testis because the gubernaculum is long allows the testis (\pm ducts) to prolapse into the groin (Adapted from Hutson *et al.*, 1997).

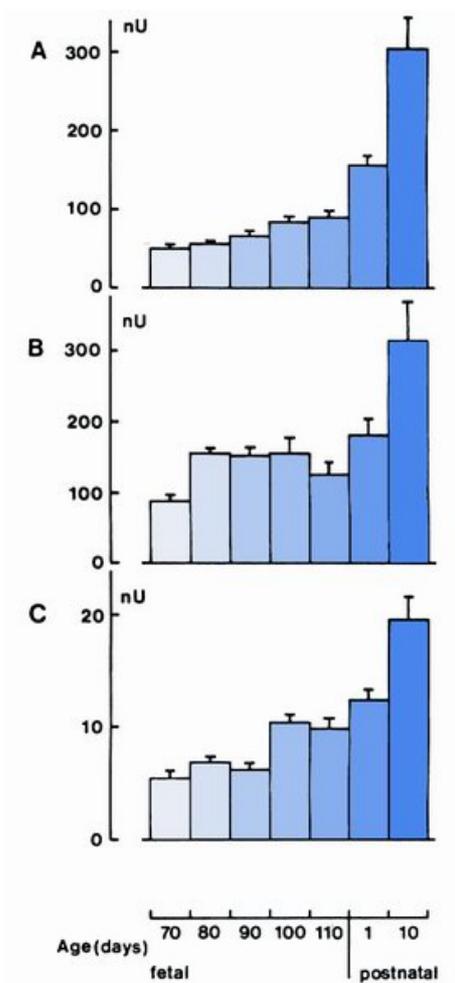


Figure 3a: Hyaluronidase activity (nU) in gubernaculum from male porcine fetuses and neonates of various ages, expressed per gram of wet tissue (A), per mg of DNA in same sample (B), or per mg of extracted protein (C); (Adapted from Fentener van Vlissingen *et al.*, 1989).

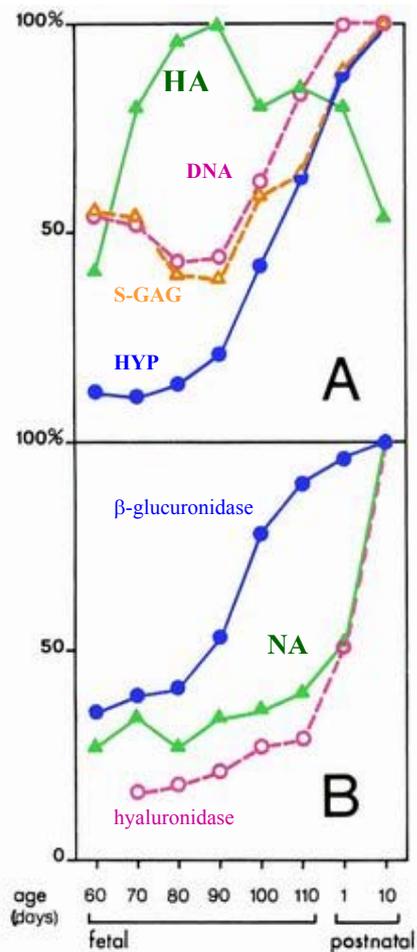


Figure 3b: Relative changes of ECM constituents and DNA (A) and acid hydrolase (B) per mg of gubernaculum testis tissue during testicular descent. Metric quantity corresponds to 100%; (Adapted from Fentener van Vlissingen *et al.*, 1989).

3.3 MOLECULAR ANALYSIS OF HYALURONIDASE GENES

3.3.1 HYAL GENES

The hyaluronidase genes are also referred to as hyaluronoglucosaminidase (*HYAL*). There are six paralogous *HYAL* genes known sharing about 40% identity with one another. *HYAL* genes are reported in the tumor suppressor gene region (*TSG*) on HSA3p21 (Wei *et al.*, 1996) and on HSA7q31 (Csoka *et al.*, 1999). This arrangement is due to an original ancient cluster formation, followed by *en masse* duplication (Stern and Csoka, 2000). On HSA3p21.3, *HYAL1*, 2 and 3 (also referred to as *Lung Cancer1*, 2 and 3) are located in a successively narrowed critical gene region, first, to ~370-kb, and, more recently, to ~120-kb containing the genes *CACNA2D2-PL6-101F6-NPRL2-BLU-RASSF1-FUS1-HYAL2-HYAL1-FUS2-HYAL3-IFRD2-SEMA3B-GNI2-G17-GNAT1-SEMA3F-G15-G16* (figure 4). This group is the critical tumor homozygous deletion region in lung and breast cancers (Csoka *et al.*, 2001; Kreil 1995; Shuttleworth *et al.*, 2002; Lerman and Minna 2000). Ji *et al.* (2002) reported that the genes act together to suppress tumor growth through their functional activation of tumor suppressing pathways. The genes' orthologs in mouse are *Hyal1*, *Hyal2*, and *Hyal3* which have recently been cloned on mouse chromosome 9F1-F2 (Shuttleworth *et al.*, 2002).

The second group of hyaluronidase genes *HYALP1*, *HYAL4* and *SPAM1* is clustered on HSA7q31.3. *HYALP1* gene represents an expressed pseudogene, current knowledge indicates that the *HYAL4* gene encodes a chondroitinase without any known activity against hyaluronan (Csoka *et al.*, 1999). The third gene in this cluster *SPAM1* encodes for protein PH-20, an acrosome intact protein. In mouse the chromosomal localization of the second cluster was reported with *6A2* (Stern and Csoka, 2000).

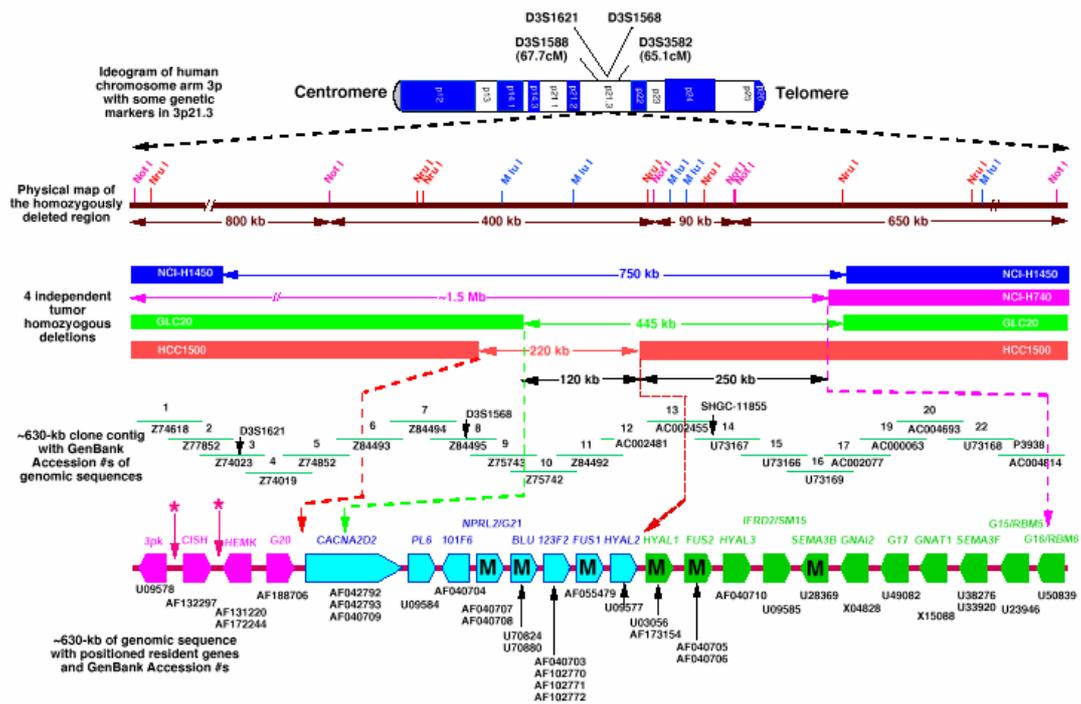


Figure 4: Genetic and physical characterization of the nested homozygous deletion region, lung cancer TSG, provisionally called *LUCA* on HSA3p21.3. The 630-kb region contains a set of 25 genes identified: 19 of these lie within the 370-kb region and 8 within the nested 120-kb region (blue boxes). The 6 genes outside of the critical region in the centromeric portion are in magenta while the remaining 11 genes still within the rest of the lung nested homozygous deletions (an additional 250 kb) are all in green. The six genes that exhibit at least some point or small mutations have an "M" in their box (Lerman and Minna, 2000).

Expression studies of the human *HYAL* genes revealed unique tissue-specific patterns. Northern blots showed that *HYAL1* and 2 are widely expressed. *HYAL3* is differentially expressed in bone marrow and testis while *HYAL4* is differentially expressed in placenta and skeletal muscle. *PH20* was detected only in testis. For *HYALP1*, the signals found in thyroid and adrenal glands after a long exposure time demonstrate that it is an expressed pseudogene (Csoka *et al.*, 1999). In mouse, expression profiles of *Hyal1*, *Hyal2* and *PH20* are similar to human but mouse *Hyal3* was broader expressed than in human. Later, reverse transcription-PCR demonstrated that some of the larger *Hyal1* transcripts seen on the Northern blot were the result of cotranscription of *Hyal1* with downstream genes, *Fus2* or *Hyal3* (Shuttleworth *et al.*, 2002). Regulation of protein synthesis is a multi-component and multi-step process involving protein, RNA and low

molecular weight ligands. Protein: RNA-interactions are dependent on the sequence and structure of mRNA. Under different physiological conditions which may be influenced by the cellular environment, virus infection or the stage of cell cycle, an mRNA may be either selectively translated or stored as an inactive protein: RNA complex (mRNP) (Woodley, 2002). Regulation of hyaluronidase activity is due to alternative mRNA splicing. The RT-PCR analysis on RNA extracted from a bladder cancer cell line, bladder tissues, lymph nodes positive for tumor, and normal kidney tissue showed alternative splicing of HYAL1 and HYAL3 in control of HYAL activities (Lokeshwar *et al.*, 2002).

3.3.2 Genes of the human hyaluronidase gene cluster on chromosome 3q21.3

1) HYAL1

The human *HYAL1* gene has been described as plasma hyaluronidase encoding 435 amino acids (aa) (Genbank Acc. No: AAD04190); Frost *et al.*, 1997) while the mouse *Hyall* codes for a protein of 462 aa (Genbank Acc. No: AAC15949; Csoka *et al.*, 1998) that is 79% identical to the human sequence. The human gene spanning ~3.5-kb contains 4 exons coding for a 2.6 kb mRNA is well expressed in all analyzed human tissues including lung and is abundantly represented in EST databases from normal and tumor tissues (Lerman and Minna, 2000). The tissue expression profile for HYAL1 was similar in mice and human with highest levels of expression in liver followed by kidney and skin (Csoka *et al.*, 1998; Shuttleworth *et al.*, 2002).

HA in prostate cancer increased 3-8-fold compared to normal tissues. The stromal-epithelial HA and HYAL1 expression may promote angiogenesis. High HA was observed in tumor-associated stroma and HYAL1 in tumor cell increases with tumor growth and metastasis (Lokeshwar *et al.*, 2001). Thus, HYAL1 is the major tumor-derived HAase expressed in bladder and prostate cancer tissues. It has a optimum pH range of 4.0-4.3, and the enzyme is 50-80% active at pH 4.5 (Lokeshwar *et al.*, 2001).

Several mRNA splice variants (figure 5) were later found in bladder and prostate cancer cells and in bladder and kidney tissues (Lokeshwar *et al.*, 2002). The authors describe finally the *HYAL1* wild type (wt) cDNA and a total of five variants (vt) encoding proteins of 405, 253, 209, 176, and 96 aa. Common for the six cDNAs is the spliced out region between nucleotides, 110 to 596 whereas the differences between variants are due to other splice sites throughout the cDNA. Further experiments with respect to HAase activity revealed no activity for variants 3, 4, and 5. Measurable HAase activity showed HYAL1 wt, but vt 1 lacking only 30 aa (compared with wt) has no activity. The vt 2 protein on the

other site shows a reduced activity of 90% compared with the wt. Lokeshwar *et al.* (2002) conclude therefore that the 30 aa sequence between aa 300 to 331 may be crucial for HA activity. They also postulate that expression of functional active HAases in different tissues is regulated by alternative splicing and they stress that expression of the wt cDNA in high grade bladder tumor tissues and as well as in invasive bladder cancer cells makes the HAase as a marker for tumor progression regardless of its grade.

Another effect of mutations in the *HYAL1* gene was described by Triggs-Raine *et al.* (1999) who identified two mutations in the *HYAL1* alleles of a patient with a deficiency of serum hyaluronidase designated as mucopolysaccharidosis IX. A 1412G \rightarrow A mutation introduces a nonconservative amino acid substitution (Glu268Lys) in a putative active site residue and they also found a complex intragenic rearrangement called 1361del37ins14 that results in a premature termination codon.

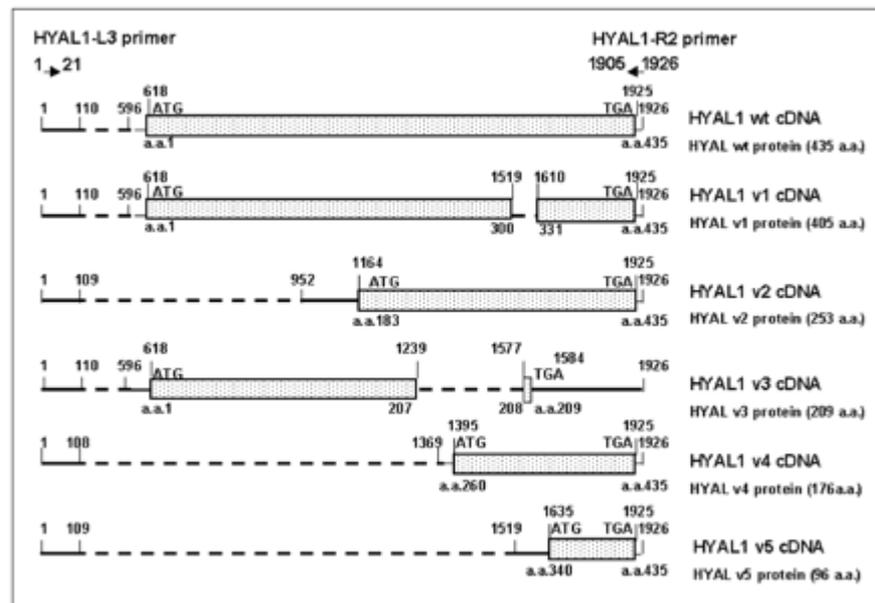


Figure 5: A schematic representation of the *HYAL1* splice variants. The figure shows *HYAL1wt* and different splice variant cDNAs. The coding region is shown as a dotted block. The dashed line shows the region that is spliced out, and solid line represents untranslated regions. Each splice junction is marked by the nucleotides at the 5' and 3' boundaries that are joined because of splicing. The translation initiation and termination codons for *HYAL1wt* and *HYAL1* variants are AF502904 (*HYAL1v1*), AF502905 (*HYAL1v2*), AF502906 (*HYAL1v3*), AF502907 (*HYAL1v4*), AF502908 (*HYAL1v5*), AF502909 (Lokeshwar *et al.*, 2002).

2) HYAL2

HYAL2 was originally designated as the lysosomal HAase as it cleaves high molecular mass HA into ~20-kDa HA fragments. It has been identified as a putative lung cancer tumor suppressor gene. *HYAL2* is not among the small subset of the 19 genes that display mutational patterns characteristic of the classical pattern of loss of tumor suppressor function (Lerman and Minna, 2000). Human HYAL2 encodes a protein of 473 aa (GenBank Acc. No: U09577). Two transcription variants of human *HYAL2* are observed in Gene Bank database, *HYAL2v1* of 1,848-bp (GenBank Acc. No: NM_003773) and *HYAL2 v2* of 1,918-bp (GenBank Acc. No: NM_033158) (Wei *et al.*, 1996). The variant v1 differs by an alternative first exon in the 5' UTR while it encodes the same protein as transcript variant 2 (v2). Human HYAL2 is expressed in many cells but not in adult brain.

The enzyme has an acidic pH-optimum below 4 with an activity that is considerably lower than observed for other types of hyaluronidases (Lepperdinger *et al.*, 1998).

The role of the lysosomal hyaluronidase on tumor invasion was investigated by overexpression of HYAL2 in astrocytoma cells. By this overexpression, intracerebral tumor growth was dramatically accelerated, but the same cells formed s.c tumors within the same time as parental cells. Thus, it seems that the interaction of the HYAL2-expressing tumor cells and the hyaluronan-containing extracellular matrix mediates this specifically in the brain (Novek *et al.*, 1999).

The ovine HYAL2 cDNA was also cloned (Dirks *et al.*, 2002; GenBank Acc. No: AF411974). The HYAL2 encoded protein functions as an efficient receptor for both Jaagsiekte sheep retrovirus (JSRV) and Enzootic nasal tumor virus (ENTV). The JSRV and ENTV envelope (Env) proteins cause cancer by inhibiting the tumor suppressor activity of HYAL2. In the mouse model, expression of human HYAL2 suppresses Env-mediated transformation by reduction of Env-protein levels by an increasing Env degradation (Liu *et al.*, 2003). The Enzootic nasal tumor virus induces nasal epithelial cancer in infected sheep. It is closely related to JSRV, which also causes cancer in sheep but in the epithelial cells of the lower airways and alveoli (Dirks *et al.*, 2002). Besides its role as a lysosomal hyaluronidase, HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for the ovine betaretrovirus JSRV that causes contagious cancers in lungs and upper airways of sheep and goats with its relationship to human bronchiolo-alveolar carcinoma (Rai *et al.*, 2001). Sheep was therefore used as an animal model, to explore the possible use of Ovine pulmonary adenocarcinoma (OPA) as a tool to investigate the mechanisms of pulmonary carcinogenesis, and to underline the similarities between the OPA and some forms of human lung adenocarcinoma (Palmarini and Fan, 2001).

3) HYAL3

HYAL3 has been detected in brain and liver tissues, its protein product is still uncharacterized. The *HYAL3* nucleotide sequence reveals that this gene contains 4 exons separated by 3 introns. Lokeshwar *et al.* (2002) described for *HYAL3* splice variants after RT-PCR in different tissues with sizes of 1.5, 1.4, 0.7, and 0.6 kb. The 1.5 kb *HYAL3* wild type (wt) was amplified from normal kidney tissue RNA, the 1.4 kb *HYAL3* variant1 (v1) from kidney tissue that is detectable in many bladder cancer cell lines. The major PCR amplification product which is present in all bladder cancer cells, tissues, and lymph node

specimens is the 0.6 kb *HYAL3* variant3 (v3) whereas the 0.7 kb *HYAL3* variant2 (v2) is present in the kidney tissue. *In vitro* translation and translation of *HYAL3*wt and variant proteins generate polypeptide chains with molecular masses of ~48, ~45, ~20 and ~16 kDa, respectively. The pH optimum of *HYAL3* HAase activities is 4.2 and only the wt is active, whereas all variants are enzymatically inactive. It is concluded that the putative catalytic site possibly Glu at position 266, and the 30 aa stretch from aa 299 to 328 are critical for HAase activity of the *HYAL3* protein. *HYAL3*v1 has no HAase activity because of the lack of the 30 aa sequence from aa 299 to 328 but it retains the putative enzyme catalytic site (Asp at position 127 and Glu at 129) and Glu at position 266 which may be generally important for HAase activity. The *Hyal3*v2 retains the 30 aa sequence and Glu266 but lacks the putative enzyme catalytic site is also enzymatically inactive. The *Hyal3*v3 that contain only Glu266 and lacks both the putative enzyme catalytic site and the 30 aa sequence has no HAase activity as well (Lokeshwar *et al.* 2002) (figure 6).

Different bladder and lymph node specimens and cells show differences in the pattern and type of *HYAL1* and *HYAL3* splice variants that are expressed. It is possible that the heterogeneous expression of the *HYAL1* and *HYAL3* variants may be related to the different biological behavior of different bladder tumors. Lerman and Minna (2000) reported that *HYAL3* RNA was not expressed in any lung cancer cell lines. Its lack of mutation and none-expression in normal lung cells makes *HYAL3* a less attractive TSG candidate.

In both *HYAL1* and *HYAL3* proteins the loss of the 30 aa sequence results in the loss of HAase activity. It is assumed that the loss of the sequence results in a loss of substrate binding as it is critical for HAase structure and activity. Studies on the *HYAL3* splice variants revealed that the 30 aa sequence is coded by one single independent exon and also that the 90-bp long independent exon (e.g missing in *HYAL1*v1) in both genes seems to encode for the protein sequence that is critical for HAase activity. The observations and the elevation of HAase levels in bladder and prostate tumors which have high levels of HAase activity suggest that *HYAL1* may play a role in promoting tumor progression. Normal cells may suppress the HAase activity of *HYAL1* by generating *HYAL1* splice variants that encode an inactive enzyme. The expression of *HYAL3*, in bladder tumor cells and tissue including prostate cancer cells reveal that the major *HYAL3* transcript is *HYAL3*v3, which is enzymatically inactive. And the minor transcript is *HYAL3*v1 (Lokeshwar *et al.*, 2002). It is possible that expression relates to the biological behavior of cancer, in carcinogenesis, biological activity and malignant potential for each

type of cancer. It may be associated with distinctive molecular and genetic alterations (Lee and Droller, 2000). Therefore, the function of these enzymatically inactive Hyal3 variant proteins remains unclear. In the mouse model, the order of the genes *HYAL2*, *HYAL1*, *HYAL3* was found to be conserved. A gene encoding an N-acetyltransferase, *FUS2*, was found completely within the first intron of *HYAL3*. Northern blot analyses of mouse hyaluronidases revealed multiple transcripts that result from alternative splicing as well as from co-transcription of *HYAL1* with *FUS2* and *HYAL1* with *HYAL3*. These complexities must be taken into consideration in strategies for the development of mouse models of hyaluronidase deficiency and in understanding the roles of these genes in health and when mutated (Natowicz and Triggs-Raine, 2001).

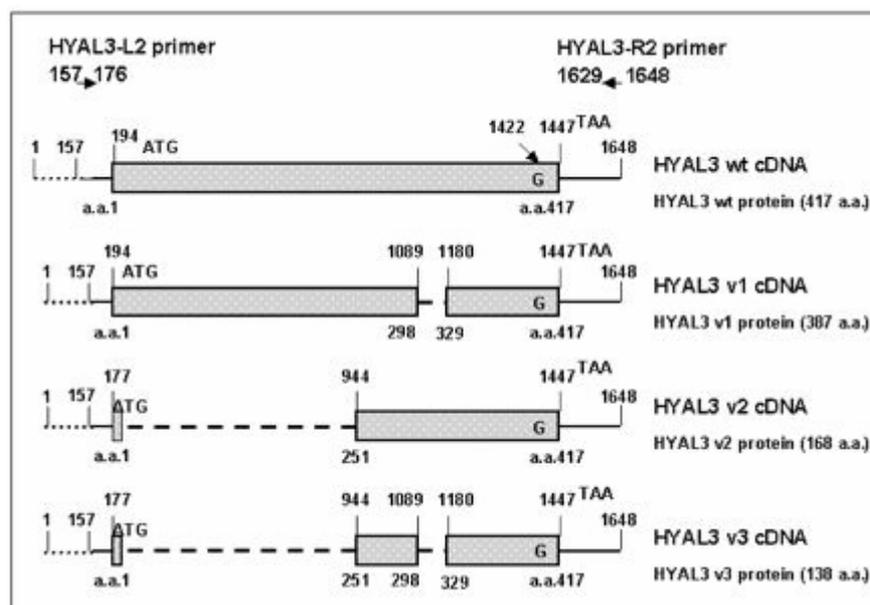


Figure 6: A schematic representation of the *HYAL3* splice variants. The figure shows *HYAL3wt* and different splice variant cDNAs; AF502909 (*HYAL3v1*), AF502910 (*HYAL3v2*), AF502911 (*HYAL3v3*), and AF502912 (*HYAL3wt*). The coding region is shown as a dotted block. The dashed line shows the region that is spliced out, and the solid line represents untranslated regions. Each splice junction is marked by the nucleotides at the 5' and 3' boundaries that are joined because of splicing (Lokeshwar *et al.*, 2002).

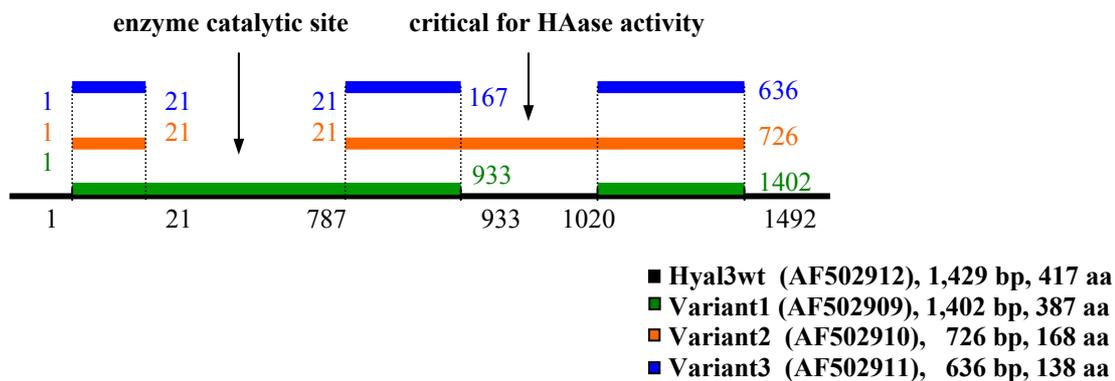


Figure 7: *HYAL3* alternative splicing of a single independent exon for control expression and enzymatically active hyaluronidase.

4) Lung cancer candidate (*FUS1*) gene

The *FUS1* gene was discovered experimentally by screening cDNA libraries with a genomic fragment from cosmids *LUCA12* and *LUCA13*. Human *FUS1* is coding for a 1691-bp mRNA (Genbank Acc. No: AF055479) with a 110-aa protein (Genbank Acc. No: NP_009206). The gene space is about 3.3-kb separated in 3 exons. The gene name "*FUS*" was given as the sequence acted as fusion between Luca12 and Luca13 at the time of its isolation. Expression of the gene by Northern Blot displayed a 1.8-kb transcript strongest expressed in heart, lung, skeletal muscle, kidney and pancreas (Lerman and Minna, 2000). Mutation of *FUS1* is infrequent in lung cancers but there is no evidence for *FUS1* promoter region methylation. Overexpression of the *FUS1* gene leads to G1 arrest and growth inhibition of lung cancer cells (Kondo *et al.*, 2001). The *FUS1* protein shows to inhibit the growth of tumors and greatly reduces the spread of lung cancer in mice. Thus, this gene may provide a novel method of selectively destroying cancer cells in humans. *FUS1* was also used in gene transfer for the Non-Small-Cell Lung Cancer, drug DOTAP; Cholesterol-Fus1 (Anderson, 2003).

5) Putative tumor suppressor gene FUS2

The FUS2 gene was discovered experimentally by screening cDNA libraries with a genomic fragment from cosmid *LUCA14*. It is an intronless single copy gene coding for a 1.9-kb mRNA (GenBank Acc. No: AF040705) expressed in normal human tissues including lung. An alternatively spliced form (Genbank Acc. No: AF040706) exists that results in the same predicted amino acid sequence (Lerman and Minna, 2000). The mouse ortholog was discovered in mouse EST databases (Genbank Acc. No: AA051756, AA051686, AI425576, AA833145). The gene product encodes N-acetyltransferase 6 and *FUS2* is therefore alternatively called Nat6 (EC 2.3.1). Computer analysis predicted the existence of the catalytic domain specific for acetyltransferases (GNAT) and a proline rich domain in *FUS2*. *FUS2* localizes to the cytoplasm and functions to acetylate the N-terminus of proteins using a ping-pong mechanism with specificity for substrates (Zegerman *et al.*, 2000). Mouse *FUS2* has been reported to be in cotranscription with *HYAL1* and *HYAL3* genes but the tissue distribution of *FUS2* and *HYAL3* in mouse differs from that in humans (Shuttleworth *et al.*, 2002). Defects in Nat6 are found in non-small cell lung cancer (nscl) cell lines. Missense mutations were detected in lung cancer cell lines that makes *FUS2* an attractive candidate for future *TSG* functional studies (Lerman and Minna, 2000; Wei *et al.*, 1996).

The *FUS2* gene is also well characterized in yeast (*Saccharomyces cerevisiae*), as a nuclear fusion protein. Its functions in promoting cell fusion during zygote formation at optimum pH 9.2 (GeneDB; YMR232W). Cell fusion is the process by which the plasma membranes of two cells fuse to establish cytoplasmic continuity. Fusion events mediate housekeeping functions such as endocytosis, constitutive secretion and also occur in special cells as in regulated exocytosis of hormones. Fusion events are also associated with disease states: the formation of giant cells during inflammatory reactions, or the entry of all enveloped virus into host cells. In many fusion events, the leaflets that face to the cytoplasm make the initial contact; this category encompasses fusion of intracellular macromolecular carrier vesicles with their target organelles. As an example the protein responsible for influenza virus fusion is a characterized hemagglutinin. A combination of factors has contributed to the prominence of hemagglutinin-mediated fusion as a model membrane fusion system (shown in figures 8A, B) (White, 1992). The mating pathway in yeast provides a model for signaling-controlled changes at the cell surface (Alia and Marsh, 1998) as well as the process by which two haploid cells fuse to form a diploid zygote (Gammie *et al.*, 1998) that can be used in sperm-egg interaction model. The fusion

of egg and sperm to form a zygote and the fusion of muscle cell precursors to generate multinucleate syncytia of muscle fibers are two examples wherein cell fusion is a key process. Mutations of *FUS2* showing to be specifically defective in cell fusion, appear to be resulting from remodeling their cell wall. The mutant also has strong defects in karyogamy and fails to orient microtubules between parental nuclei in zygotes (Elion *et al.*, 1995).

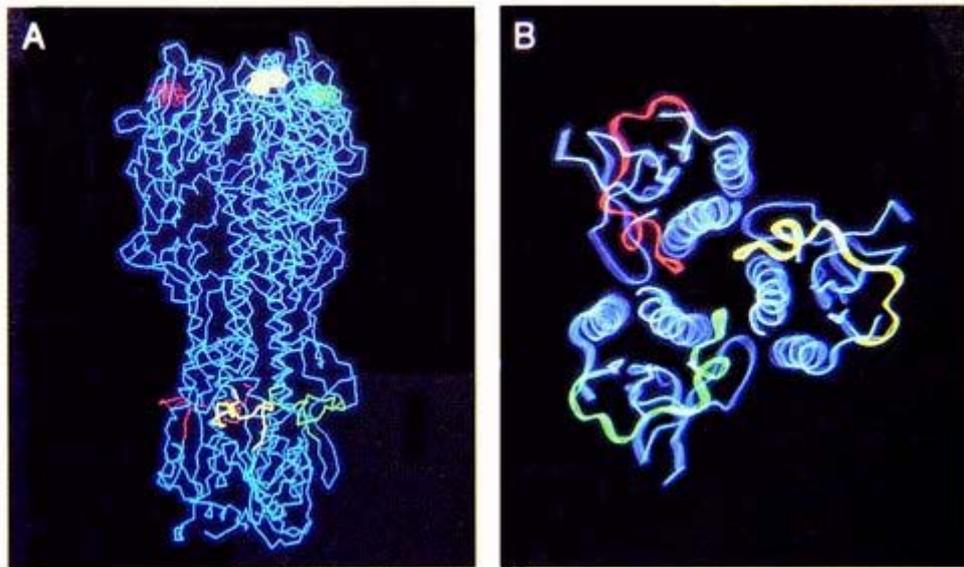


Figure 8: The influenza hemagglutinin. The carbon backbone of the hemagglutinin trimer is displayed with the three fusion peptides, located in the stem of the molecule and molecular surfaces of sialic acid residues are shown in the receptor binding sites if the globular head domains (highlight in red, yellow and green) (figure A). Figure B shows ribbon diagram of the hemagglutinin trimer cross section highlighting the three fusion peptides (Adapted from White, 1992). This model has suggested potential fusion proteins and several of this assignment have been supported by site-specific mutagenesis.

3.4.2 Associations between hyaluronidases and pigs

Hyaluronidase research activities in the pig area focus on food safety control against bacteria, nematodes and denuded oocytes and for *in vitro* fertilization (Swain and Krisher, 2002). In medical research, studies of hyaluronidase in the porcine aortic heart valve were done to use the results in heart valve replacement surgery. It was hypothesized that the enzymatic mechanisms may be responsible for glycosaminoglycans within the extracellular matrix of bioprosthetic heart valves degeneration (Simionescu *et al.*, 2003).

The nematode *Ascaris suum* is a threat to public health because the worm is infectious for humans as well (Urban *et al.*, 1998). Hyaluronidase is released by *A. suum* and is required for gastrointestinal mucosal invasion (Hotez *et al.*, 1992). The worm is responsible for significant economic losses by reducing production efficiency and causing organ condemnations at slaughter due to pathology produced by larval stages L3 and L4 (Rhoads *et al.*, 2001). Surveys in a organic pig herd recorded in a period from March to October 1999 showed that the pigs were naturally infected with *A. suum* (28% of weaners, 33% of fatterners, 14% of saws). The *Ascaris* infective eggs were found in 14% of solid sample from saw pastures and in 35% from slaughter pig paratus (Carstensen *et al.*, 2002). Furthermore, chickens and pigs can be the transporting host for the human parasite equivalents (Olsen *et al.*, 2001). Thus, the infections of roundworm depends on many factors such as environmental conditions larval development in the egg and egg survival, age of the host, breed, husbandry system, hygiene and treatment schedule (Okulewicz *et al.*, 2002).

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