Immunohistochemical localization of PAX7, AE1+AE3 pankeratin, SOX2 and neurofilament protein in the early porcine embryo

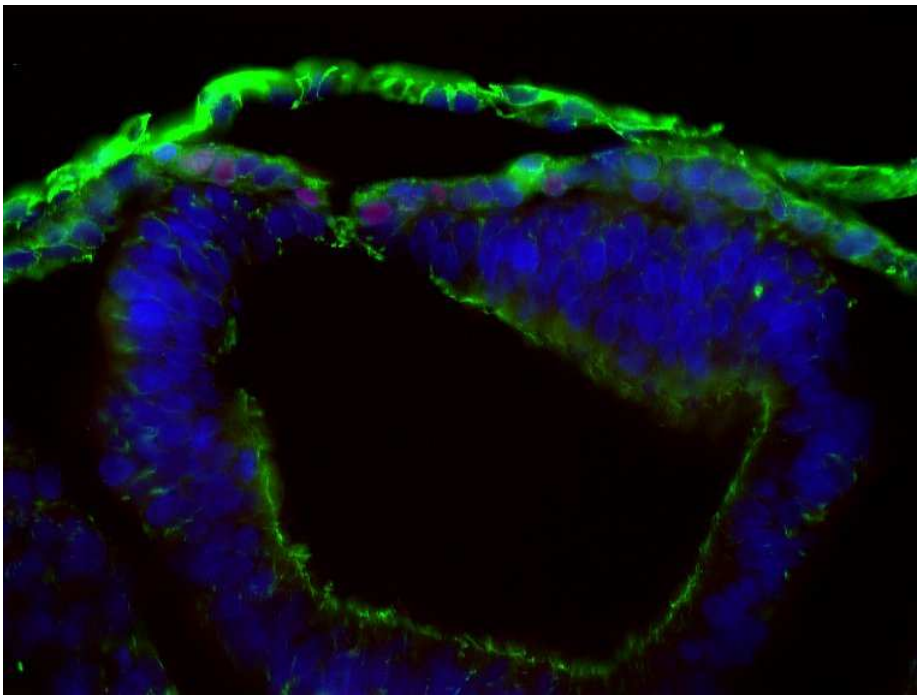

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Photo on front page: The closing neural groove seen in a transversely sectioned *neural groove* stage embryo. (x 64). Monoclonal mouse anti chicken PAX7 1:5 (Alexa 594) and monoclonal mouse anti human pankeratin 1:200 (Alexa 488).

Der findes én visdommens vej –
det er den, som bør være let at erindre:

Dum dig
og dum dig
og dum dig igen,
men mindre
og mindre
og mindre

Piet Hein

The road to wisdom? - Well, it's plain
and simple to express:
Err
and err
and err again
but less
and less
and less.

Piet Hein

Preface

This veterinary thesis has been conducted at the Royal Veterinary and Agricultural University of Denmark, Department of Basic Animal and Veterinary Sciences, Anatomy and Cell Biology, as a part of the Doctor of Veterinary Medicine program.
The thesis is written in English in the form of a scientific article. It is targeting veterinarians, scientists and others that have interest in the field of neurulation and embryological development.
The projects aim is to characterize localization of PAX7, SOX2, NF-protein and AE1+AE3 pankeratin in the early porcine embryo. Little is known on porcine markers at this time.
My thanks go to the staff at the Department of Basic Animal and Veterinary Sciences, Anatomy and Cell Biology, who has been very willing to assist in the project. From the technical staff especially Hanne Holm has been very patient with my questions and requirement for introduction to new methods in the laboratory. Gunnel Holden has helped me out when time came short and given good pieces of advice.
From the scientific staff Morten Vejlsted and Esben Østrup have been very willing to discuss the project. They have given many helpful pieces of advice for which I thank them. Lars-Inge Larsson needs mentioning after bringing a perspective of the obstacles scientists face on an everyday basis into this project.
Kirsten Schauser has been more than helpful. She has always taken the time to help me and to discuss any problems met along the path of the project. This was much more than I ever expected, thus she has my deepest appreciation. With Poul Maddox-Hyttel there has been several meetings when things did not work out as planned. It has always been with his enormous breadth of view and amazing knowledge for even the smallest details that this project has been furthered and solutions have been addressed. Last I would like to thank my wife Thea for being so patient and supportive of me during the work of the project.

Frederiksberg, 28 August 2006

Kristian M. Kamstrup

Resumé

Det var projektets mål at karakterisere lokalisationen af PAX7, SOX2, AE1+3 pankeratin and neurofilament protein I den tidlige udvikling af det porcine nervesystem.

Der blev anvendt 10 svineembryoner i alderen 10-20 dage i forsøget. Immunhistokemisk farvning blev brugt til at finde ud af om der var ekspression af PAX7, SOX2, neurofilamenter og AE1 og AE3 pankeratin.
De 10 embryoner var først gruppeinddelt efter stadier ved hjælp af et stereomikroskop. De blev gruppeinddelt i følgende 4 stadier: *præ-primitivfure*-stadiet der indeholdt 3 embryoner, *neuralfure*-stadiet der indeholdt 3 embryoer, *somit*-stadiet der indeholdt 2 embryoner og *lemmeknop*-stadiet der indeholdt 2 embryoner.

Udtryk af PAX7 kunne ikke erkendes på *præ-primitivfure*-stadiet og *somit*-stadiet. Der sås PAX7-farvning i *neuralfure*-stadiet, hvor formodede *crista neuralis*-celler var mærkede for PAX7 immunfarvning. I *lemmeknop*-stadiet sås PAX7 udtrykt i dermomyotomer samt i det dorsale neuralrør.

SOX2 kunne ikke erkendes på *præ-primitivfure*-stadiet, *neuralfure*-stadiet og *somit*-stadiet. SOX2 blev udtrykt cytoplasmatisk i celler i neuralrøret ved embryoner på *lemmeknop*-stadiet.

Der kunne ses farvning for AE1+AE3 pankeratin i epiblast og hypoblast på *præ-primitivfure*-stadiet. På *neuralfure*-stadiet, *somit*-stadiet sås ektoderm og endoderm. På *lemmeknop*-stadiet kunne AE1+AE3 pankeratin erkendes i ektoderm og endoderm. Desuden sås ekspression i notochordaen.

Mærkning for neurofilamentprotein kunne ikke erkendes på *præ-primitivfure*-stadiet, *neuralfure*-stadiet og *somit*-stadiet. Der blev set mærkning for neurofilamentprotein på *lemmeknop*-stadiet.

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1 Abbreviations

BMP	Bone morphogenic protein
Cdx	Caudal-type homeobox protein
CNS	Central nervous system
d.p.i.	Days post insemination
DNA	Deoxyribonucleic acid
FGF	Fibroblast growth factor
H3K9me2	Histone H3 lysine 9 dimethylated
H4	Histone H4
HMG	High-Mobility-Group
ICM	Inner cell mass
IgG	Immunglobulin G
IgM	Immunglobulin M
In vitro	Within a glass
In vivo	Within the living body
KVL	Royal Veterinary and Agricultural University
NF	Neurofilament
OCT4	Octamer-binding transcription factor 4 protein
PAX7	Paired box gene 7
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
SOX2	*Sry*-related HMG-box containing genes
WNT3a	Wingless-type MMTV integration site fam., memb. 3A

2 Abstract

The project's aim was to characterize the expression of PAX7, Sox2, AE1+3 pankeratin and neurofilament protein with respect to localization in the early development of the porcine nervous system.

Using 10 porcine embryos ranging from 10 to 20 days, immunochemistry was used in this thesis to reveal the expression of the transcription factors PAX7 and SOX2, the intermediate filaments expressed in neurons, known as neurofilaments, and finally a marker for AE1+AE3 pankeratin.
The 10 embryos were first stereomicroscopically staged which distributed them into the following 4 groups: a *pre-streak* stage group containing 3 embryos, a *neural groove* stage group containing 3 embryos, a *somite* stage group containing 2 embryos and a *limb bud* stage group containing 2 embryos.

Expression of PAX7 was not seen at the *pre-streak* stage or at the *somite* stage. It was seen expressed in assumed neural crest cells nuclei at the *neural groove* stage. At the *limb bud* stage expression was seen at the dorsal neural tube and in the dermomyotomes.

SOX2 was not expressed in the *pre-streak* stage embryos, the *neural groove* stage embryos or the *somite* stage embryos.
SOX2 was expressed in the neural tube of the embryos at the *limb bud* stage.
Cytoplasmic expression of AE1+AE3 pankeratin was seen in the epiblast and hypoblast of *pre-streak* stage embryos. In the *neural groove* stage embryos and in the *somite* stage embryos it was seen in ectoderm and endoderm. At the limb bud stage it was seen expressed in the surface ectoderm, the endoderm, and it was seen expressed in the notochord.
The occurrence of neurofilament proteins was not found in *pre-streak* stage embryos, the *neural groove* stage embryos or the *somite* stage embryos. It was seen in *limb bud* stage embryos.

3 Introduction

In order to understand diseases in humans better, pigs are being produced in expectation that they will prove to be better models of human disease than the current rodent models. Pigs have a more comparable size to humans, and a similar metabolism and heart frequency than do rodents. Their gross brain anatomy is also more similar since pigs have a gyrencephal brain as opposed to the lisencephal brain of rodents. The production of genetically modified pigs using nuclear transplants has already been initiated. The first pigs are meant to be born in October 2006.

In order to allow for parallel *in vitro* testing, a concurrent cell lineage will be made. This is meant to be done by from genetically modified embryos where experiments are aiming to isolate and grow porcine embryonic stem cells. These embryological stem cells are thought to be stimulated to differentiate into e.g. nerve cells. To be able to identify the different cell types, ways to identify the cells have to be found. To do this normal porcine embryos are used and labelled immunohistochemically, in order to find relevant transcription factors, receptors and intracellular proteins characterizing porcine neuronal development that later can be applied to the identification of cultured nerve cells.

Using 10 porcine embryos ranging from 10 to 20 days, immunochemistry was used in this thesis to reveal the expression of the transcription factors PAX7 and SOX2, the intermediate filaments expressed in neurons, known as neurofilaments, and finally a marker for AE1 + AE3 pankeratin. This will be addressed later.

3.1 Embryonic development

3.1.1 Blastulation in pigs

The zygote, the fertilized oocyte, starts cleaving through mitotic division increasing the number of cells. These cells are known as blastomeres. The blastomeres are totipotent which means that they have the ability to become extraembryonic as well as intraembryonic tissue. During compaction there is a segregation of the outer cells in the blastula which are combined by tight junctions and the inner cell mass segregates from them. This is a differentiation of the blastomeres into precursor cells to trophoblast and inner cell mass cells. Fluid enters the cell mass and a cavitation is formed known as the blastocele. The inner cell mass cells (ICM) retain the ability to differentiate into the different cell types in the embryo except trophoblast cells, which is known as pluripotency (Figure 3.1).

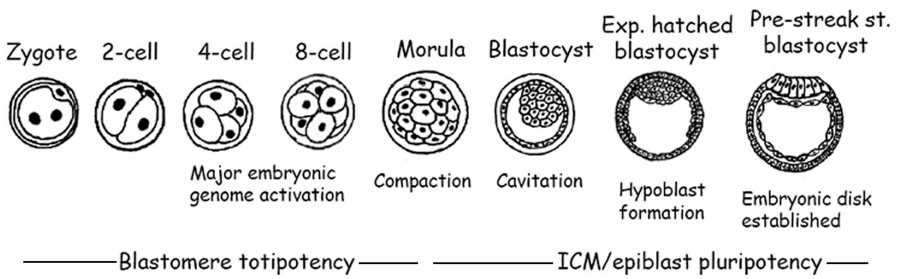

Figure 3.1 The early embryonic development from the zygote to the *pre-streak* stage blastocyst. The horizontal lines indicate where the cells are believed to go from totipotent to pluripotent stages (Vejlsted, 2006).

The transcription factors OCT4, SOX2 and Nanog regulate the expression of other genes during early development.

OCT4 is a transcription factor required for pluripotency. It belongs to the POU factor family binding to the octamer motif ATGC(A/T)AAT in regulatory domains of many genes (Cavaleri and Schöler, 2004).

SOX2 is a transcription factor in the SOXB1 subfamily which means it has a great similarity to SOX1 and SOX3 sharing 90 % of the amino acid sequences of their HMG-DNA binding domain (Carlin *et al.*, 2006). HMG stands for High-Mobility-Group. It is a peptide with an amino acid sequence similar to the HMG-box of *Sry* (the sex-determing regions of the Y-chromosome). It is seen e.g. in Sox-genes. Sox means *Sry*-related HMG-box containing genes (Pevny and Lovell-Badge, 1997). SOX2 is co-expressed with OCT4 and evidence suggest they have a synergistic action (Cavaleri and Schöler, 2004).

Nanog is a homeodomain transcription factor seen in pluripotent cells. It is an *in vitro* marker for all pluripotent cell lines (Cavaleri and Schöler, 2004). Nanog has been reported to block bone morphogenic protein-induced differentiation of embryonic stem cells (Suzuki *et al.*, 2006).

When pluripotent cells differentiate to somatic cells and also during gastrulation, OCT4 is down regulated (Vejlsted *et al.*, 2006b;Pesce and Scholer, 2000). High levels of OCT4, SOX2 and Nanog are then only found in the cells of the inner cell mass and not in the trophectodermal cells (Figure 3.2). The down regulation of these transcription factors is in correlation with the loss of pluripotency and the ability to self-renewal characterizing stem cells, and it shows the beginning towards cellular differentiation (Carlin *et al.*, 2006;Ralston and Rossant, 2005).

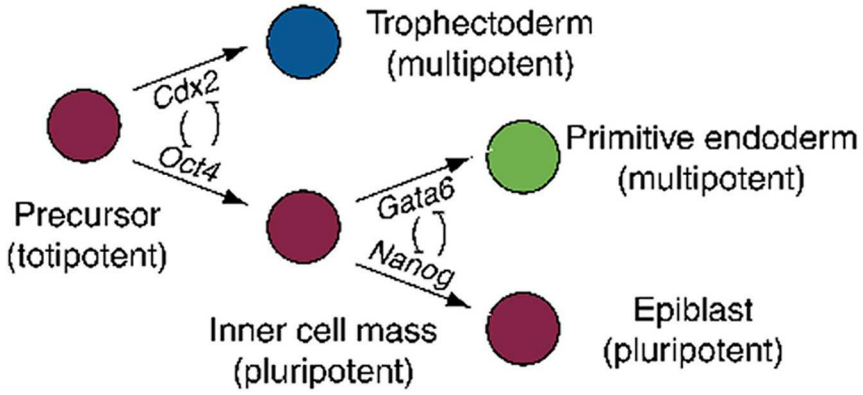

Figure 3.2: In the mouse embryo OCT4 is considered to promote differentiation towards inner cell mass cell whereas the transcription factor, Cdx2, is considered both to restrict expression of OCT4 to inner cell mass cells and promote formation of the trophoblast. Similarly Nanog along with OCT4 later induces differentiation to epiblast cells whereas Gata6 expression will generate hypoblast (Ralston and Rossant, 2005).

More needs to be known about what controls the differentiation of blastomeres into ICM cells and trophoblast. One explanation is that it is epigenetically controlled by genomic imprinting. Genomic imprinting means that paternal and maternal DNA are marked in different ways by the means of e.g. methylation of cytosine or acetylation of histones. This makes DNA folding on histones different thereby generating areas with different readability known as exons and introns. Paternal genes are thought to regulate foetal growth while maternal genes try to spread nutrition evenly, while protecting the mother for future generations according to the "conflict theory" by Haig (Moore and Haig, 1991). The implantation of a foetus can be seen as a kind of battle between the sexes (Tilghman, 1999). It has been shown that the histones in promoter regions for Nanog and OCT4 are enriched in acetylated H4 in ICM but not in trophectoderm where the same promoter regions are enriched with H3K9me2. The converse is seen for the Cdx promoter in the trophectoderm as regards to the Cdx promoter region in the ICM. The acetylations seem to be marks for active genes, whereas the dimethylated genes are silent genes in the early embryo (O'Neill *et al.*, 2006).

3.1.2 Placentation in pigs

The trophoblast layer continues to grow making the conceptus a spherical blastocyst (Figure 5.1). Further on the epithelial cells in the trophoblast start to elongate making an ovoid blastocyst that elongates to be tubular ending up as a 1 meter long filamentous gastrula. The

membranes are heavily folded leaving the embryo occupying only 10-15 cm (Nielsen, 1965).

In pigs implantation begins on day 13-14. The porcine placenta is non-deciduate and epitheliochorial (Noden, 1985;Dantzer and Leiser, 1998;Dantzer, 1985) the foetal membranes are easily separated from the endometrium. This kind of implantation is in contrast to the interstitial attachment preceding the hemochorial placenta seen in man (Sadler, 2006). Therefore the term “initial attachment” is probably more correct than implantation. The initial attachment is often concomitant with gastrulation (Noden, 1985).

3.1.3 Gastrulation in pigs

In pigs the embryonic disc develops during the blastocyst stage. It is first seen as a condensation of the trophoblast called the inner cell mass (ICM). The overlying trophoblast, also known as Raube’s layer, eventually degenerates after hatching from zona pelucida. This exposes the inner cell mass to the surface of the blastocyst, thereby renaming it to the “embryonic disc”.

As the first event in gastrulation, cells delaminate from the inner surface of the ICM, generating the hypoblast that eventually covers the inside of the trophoblast. The lumen enclosed by the hypoblast is referred to as the primitive gut, archenteron, thus the name gastrulation (Noden, 1985;Nielsen, 1965). A crescent shaped thickening of the posterior embryonic disc marks the beginning delamination from the embryonic disc. The delamination is the process where cells stemming from the embryonic disc start to give rise to the endoderm and early mesodermal cells. Eventually the hypoblast cells are supplanted by endodermal cells. Concurrently the lengthwise growth of the embryonic disc is in posterior direction thus dragging out what is known as the primitive streak in the epiblast. In the anterior streak the primitive node is formed. Epiblastic cells move medially towards the primitive streak, involute, and spread underneath the epiblast generating the mesoderm as well as the endoderm. As the mesoderm grows anteriorly the primitive streak and node moves posteriorly, which stretches the notochord in the length of the embryo (Nielsen, 1965;Noden, 1985;Rüsse and Sinowatz, 1991).

3.1.4 Neurulation

Neurulation in the pig starts when the primitive streak regresses caudally and the cranial epiblast transforms into the neural plate. The epiblastic transformation into the neural plate is induced by the anterior chorda-mesoderm-complex also known as the pre-chordal plate

(Rüsse and Sinowatz, 1991) (Figure 3.3). This is according to the "default model" (Hemmati-Brivanlou and Melton, 1997) due to expression of chordin, noggin, and folistatin from the pre-chordal plate which antagonizes bone morphogenic protein (BMP) in the cranial region of the embryo (Kintner and Koyano-Nakagawa, 2004;Weinstein and Hemmati-Brivanlou, 1999). If BMP becomes inactivated ectoderm becomes neuralized (Sadler, 2006). BMP-4 is inactivated in the hindbrain and spinal cord region by WNT3a and FGF (Sadler, 2006;Wilson and Edlund, 2001) and SOX2 is expressed through activation of SOX2 enhancer N-1 (Takemoto *et al.*, 2006). A significant expression of SOX2 has been shown to maintain neuronal progenitor characteristics, thereby inhibiting the progenitor cells final differentiation towards neurons. SOX2 is expected to be seen in neural progenitor cells (Carlin *et al.*, 2006).

The epiblastic differentiation towards neuronal precursor cells is reported as early as the blastula stage (Wilson and Edlund, 2001).

The neural plate is at the three somite stage wide cranially, narrow at the somite level and widen again caudally. Eventually the lateral edges of the neural plate become enlarged and elevated forming the neural folds (Figure 3.3). Coincidentally the neural groove appears medially in the neural plate. The neural folds start to appose each other and eventually start fusing (van Straaten *et al.*, 2000;Sadler, 2006). There are 3 initial closure sites at the 8 somite level, leaving four temporary neuropores. At the 10 somite level the neural tube has closed the two middle neuropores and closure continues bidirectionally. The caudal neuropore closes as the last around the 28 somite stage (van Straaten *et al.*, 2000)(Figure 3.4 A).

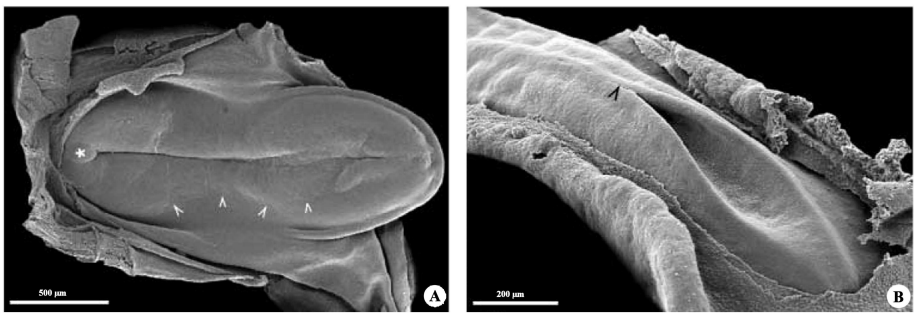

Figure 3.3 A-B: A) The border of the neural plate (arrowheads) as well as the pre-chordal plate (asterisk) are seen at the 3 somite stage of a porcine embryo. Bar 500 µm. B) The zipper-like closure of the neural groove with the caudal part is still open is seen at a 13 somite stage porcine embryo. The raphe (arrowhead) between the apposing neural folds is seen dorsally. Bar 200 µm. Scanning electron microscopic images (van Straaten et al., 2000).

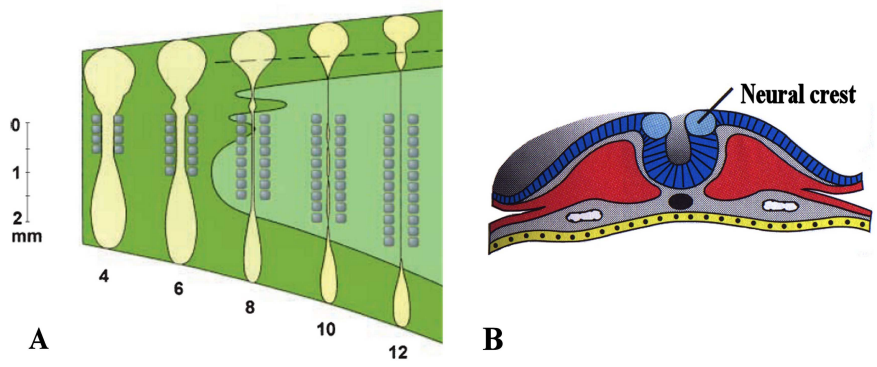

Figure 3.4 A-B: A) Schematic reconstruction of neurulation. The neural plate and neuropores are coloured yellow while the not fusioned apposing neural folds, the raphe, is drawn as a narrow yellow zone. Progression of closure is indicated by the curved borderline between the light and dark green areas. The mesencephalic flexure (dotted line) is drawn as being not bent (van Straaten *et al.*, 2000). B) Schematic reconstruction of the apposing neural folds in the closing neural groove (blue) with the position of the neural crest cells indicated (Sadler, 2006).

3.1.5 Neural crest development

The neural crest is the name for the lateral border of the folding neuroectoderm, consisting of a cell population that will leave the neuroectoderm through active migration (Figure 3.4 B). The neural crest cells give rise to numerous cell types in the animal among others to melanocytes, sensory ganglia, sympathetic neurons, glial cells and Schwann cells (Sadler, 2006). Previously thought to be induced by interactions with mesoderm and non-neural ectoderm, it has been shown that neural crest induction happens well before the appearance of the neural plate and that it requires the transcription factor PAX7 (Basch *et al.*, 2006). PAX7 is a transcription factor containing 128-amino acid DNA-binding domain, called the paired domain. Pax genes are important regulators of organogenesis in all species being a group of transcription factors that have been conserved phylogenetically for millions of years (Chi and Epstein, 2002). The identification of PAX7 expression has yet not been seen on porcine embryos.

Neurofilaments (NF) are only seen in neurons (Liu *et al.*, 2004). They belong to the neurons cytoskeleton as members of the group of intermediate filaments. More specifically they are intermediate filament of type IV that are prominent in neuronal, immature neuronal and CNS stem cells (Liu *et al.*, 2004). There are again 3 types of NF proteins of different weight that the neurofilaments are composed of. They are synthesized in the perikarya and transported in the axons along the

microtubules. In CNS disorders such as Parkinson's disease NF subunits among other intermediate filament subunits, accumulate as neuronal inclusion affecting normal neuron function (Liu *et al.*, 2004).

Pankeratin is a marker for different keratins seen in epithelial cells. Keratin is most notably expressed in stratified squamous epithelium. The reason it was sought in this work was to try to distinguish the surface epithelium from the neuroepithelium. Keratins are intermediate filaments of the types I and II (Cooper and Hausman, 2007). The keratins investigated in this project are AE1 and AE3 pankeratin.

3.2 Aims of the study

The purpose of this study was to characterize the expression of PAX7, Sox2, AE1+3 pankeratin and neurofilament protein with respect to localization in the early development of the porcine nervous system.

4 Materials and Methods

Overall the embryos used were between 10 and 20 days post insemination (d.p.i.). They were fixed in paraformaldehyde, embedded in paraffin and coloured immunohistochemically, in order to find relevant transcription factors, receptors and intracellular proteins characterizing porcine neuronal development.

4.1 Collection, storage and selection of embryos

A total of 10 embryos were used. Of the 10 embryos used in this thesis 8 have been collected from sows with a known insemination date. They have all been collected at the Danish Crown abattoir in Skærbæk as part of the practical work behind the staging system presented in Vejlsted et al. (2006a).

The embryos were flushed out of the exvicerated uteri, photographed and fixed in 4% paraformaldehyde in 0.15 M phosphate buffered saline (PBS)(KVL Pharmacy, Frederiksberg, Denmark) for one hour. They were subsequently staged.

The embryos were after fixation stored in a 1% paraformaldehyde solution and kept at 4º C in Nunclon^{TM} Δ Surface 4-wells (Nunc, Roskilde, Denmark) for 1 year until current use.
The remaining 2 embryos were taken from the -80º C freezer originating from an older project. The embryos were taken from cut open uteri photographed and fixed in 4% paraformaldehyde in 0.15 M PBS (KVL) for one hour. The age was determined from crown-rump length (Rüsse and Sinowatz, 1991) and they were immediately thereafter frozen and kept at -80º C (Larsen, 2004).

The embryos were grouped using a 6 staged system staging system for post-hatching embryos based on stereomicroscopic appearance (Vejlsted *et al.*, 2006a). The stages are as follows: (1) Expanded hatched blastocyst stage where the embryo presents an inner cell mass covered by trophoblast. (2) Pre-streak stage I where the embryonic disc is formed. (3) Pre-streak stage II where a crescent-shaped thickening of the caudal portion of the embryonic disc appears. (4) Primitive streak stage where the primitive streak has developed as an axis of cell ingression of cells for meso- and endoderm formation. (5) Neural groove stage where the neural groove is developing from the rostral pole of the embryo along with a proportional shortening of the primitive streak; and (6) Somite stages where paraxial mesoderm gradually condensates to form somites.

Staged using stereomicroscopical appearance the selected embryos are distributed as follows:

- *Pre-streak* stage: 3 embryos
- *Neural groove* stage: 3 embryos
- *Somite* stage: 2 embryos
- *Limb bud* stage: 2 embryos

Pre-streak stage I and II have been merged into at group named *pre-streak* stage. There are no representatives from the primitive streak stage. As the real antigens were not available, the positive control was not made *lege artis*. Instead it has been assumed that the antigens were expressed at the *limb bud* stage, rendering the 2 embryos an additional role of positive control specimens.

4.2 Cryo and paraffin sections

4.2.1 Cryo sections

The embryos were oriented using a stereomicroscope and embedded in a 4% agar solution in order to ensure correct orientation when freezing them down. Following the agar embedding they were transferred to a 30% sucrose in PBS solution for 36 hours. This was done as cryoproctection to avoid tissue damage from ice crystals during freezing (Clausen *et al.*, 1991).
Embryos were placed in small trays containing Tissue-Tek® O.C.T™ Compound (Sakura) and then lowered into a tube containing cooled *n*-hexane, which was immersed in liquid nitrogen. This was done to avoid a gas pocket of nitrogen causing freezing to be so slow that tissue damage inadvertently was induced.
The frozen specimens were then placed in the cryo microtome (Leica Jung Frigocut 2800 E) oriented in a way so that the embryos were sagittally sectioned. Sections of 6 µm were cut and collected two by two on SuperFrost® Plus Microscopic slides (Menzel-Gläser, Braunschweig, Germany). Every fifth slide was only added one section and was stained with haematoxylin-eosin (Bie & Berntsen, Rødovre, Denmark) for orientation purposes.
The unstained slides were placed at -20º C for storage until needed for this project.

4.2.2 Paraffin sections

Paraffin sections proved to be unsuitable for some of the antigens used, since the immunohistochemical staining generated a considerable background stain. The use was thus only limited to the initial trials.

4.3 Immunohistochemistry

Immunohistochemical staining utilizes antibodies for detection of tissue antigens. The most frequent used antibodies are IgG and IgM (Boenisch, 2001). Antibodies can be either mono- or polyclonal. Monoclonal antibodies stem from a clone of plasma cells and react against a specific epitope on the antigen. Polyclonal antibodies are a mixture of monoclonal antibodies and are thus reacting on several different epitopes on the antigen they recognize.
The staining can be either direct or indirect. When using indirect staining the terms primary and secondary antibodies come in use. The primary antibody is the one targeting the antigen. The secondary antigen is directed against the species of the primary antibody and has flourophores or chromogenes attached to it.
The immunochemical staining method used in this project was indirect immunofluorescence. Firstly, the main advantage of this method was the fact that the signal can be amplified. Since the secondary antibody was polyclonal there could be a binding to several epitopes on the same primary antibody and thus the signal could be enhanced. Secondly, double staining can easily be achieved if the primary antibodies are from different species.
The primary antigens used in this project are all murine monoclonal antibodies. This makes double staining a little more difficult since the first primary antibody-secondary antibody binding has to be fixed and unbound primary antibodies have to be blocked. This was obtained using blocking in 4% paraformaldehyde in 0.15 M PBS for 30 min microwaving at 1500 W in 0.01 M citrate buffer (Tornehave *et al.*, 2000).

4.3.1 Antibodies used

Primary antibodies used were: monoclonal mouse anti human SOX2 (100 μg/ml; 1:100; Mab 2018, R&D Systems, Minneapolis, MN), monoclonal mouse anti human AE1+AE3 pankeratin (1:400; Uffe Birk Jensen, Human genetics, University of Aarhus, Aarhus, DK), monoclonal mouse anti chicken PAX7 (1:5; Developmental Studies Hydridoma Bank, Iowa City, IA) and monoclonal mouse anti-human neurofilament protein, clone 2F11 (1:400; M0762, DakoCytomation, Glostrup, DK). Negative control slides were incubated with nonimmune sera mouse IgG2 (0.2 mg/ml; 1:400; X0931, DakoCytomation, Glostrup, DK). The primary antibodies were diluted in 0.25% BSA (A7906, Sigma-Aldrich Danmark A/S, Broendby, DK)) in 0.15 M PBS (RVAU Pharmacy, Frederiksberg, DK). Secondary antibodies used were: Alexa 594 goat anti mouse (2 mg/mL; 1:400; A11032; Molecu-

lar Probe, Eugene, OR) and Alexa 488 goat anti mouse (2 mg/mL; 1:400; A11029; Molecular Probe, Eugene, OR).

The concentrations for AE1+AE3 pankeratin, PAX7 and NF-protein were not available.

4.3.2 Control

As the antigens were not available for the positive control of the antigens, the *limb bud* stage embryos were chosen as they were assumed to express the antigens since they were at a more advanced developmental stage than the other groups.
The lack of the antigens also applies to the negative control where no preabsorption studies could be made. Instead the immunoglobulin type of the antibodies was used as primary antibody for the negative control.

4.3.3 Method for labelling

The following double stainings were used: I) Pax7-Alexa 594/Pankeratin- Alexa 488, II) Pax7-Alexa 594/NF-protein- Alexa 488, III) Pax7-Alexa 594/Sox2- Alexa 488, IV) NF-protein -Alexa 594/ Sox2 - Alexa 488.

The slides with cryo sections were left to gain room temperature and the tissue sections were encircled with a DakoCytomation pen (S2002, DakoCytomation, Glostrup, DK) creating a hydrophobic barrier. The slides were subsequently washed in 0.15 M PBS 2×5 min. They were then incubated in 10% goat serum (X0907, Dako Denmark A/S, Glostrup, DK) in 0.25% BSA/0.15 M PBS for 30 min. The first primary antibody was added and slides were left to incubate overnight at 4^{o} C followed by 1 h at room temperature, and then washed in 0.15 M PBS 3×5 min. The secondary antibody Alexa 594 goat anti mouse (1:400; Molecular Probe) was added. Slides incubated 30 min at room temperature before washed with 0.15 M PBS 3×5 min. The slides were then treated with 4% paraformaldehyde in 0.15M PBS for 30 min, washed with PBS 2×5 min, microwaved at 1500 W in 0.01 M citrate buffer, pH 6 for 3×5 min using the method presented by Tornehave *et al.* (Tornehave *et al.*, 2000) to block remaining reactive immunglobulins.

After resting for 20 min and washed in PBS 2×5 min the slides were again incubated in 10% goat serum (X0907, Dako Denmark A/S, Glostrup, DK) in 0.25% BSA/PBS for 30 min to preblock. The second primary antibody was added and the slides were to incubate 1 h at room temperature, and then washed in 0.15 M PBS 3×5 min. The secondary antibody Alexa 488 goat anti mouse (1:400; Molecular Probe)

was added. Slides incubated 30 min at room temperature before being washed with 0.15 M PBS 3×5 min. They were then stained with Hoechst 33258 0.1 g/ml in 1 minute and wash with 0.15 M PBS 2×3 min plus short in distilled water.

The slides were subsequently mounted with DakoCytomation Fluorescent Mounting Medium (S3023, DakoCytomation, Glostrup, DK)

Microscoping was done one a Leica Leitz DMRB microscope. Acquisition of images was done using the microscopes “COHU High Performance CCD Camera” via the Leica software “QFISH V2.3b”

5 Results

In order to localize the presence of PAX7, SOX2 and the expression of AE1+AE3 pankeratin and NF-filament in the early porcine embryo a detailed study was set up. Since PAX7 and SOX2 both are transcription factors, expression of these has to be seen in the nuclei for staining to be specific. Keratins and NF-proteins are both cytoplasmic constituents therefore expected to be seen in the cytoplasm.
The time line was based on the stereomicroscopic staging by Vejlsted *et al.* (Vejlsted *et al.*, 2006a), dividing the 10 embryos into the following 4 groups: *Pre-streak* stage containing 3 embryos, *Neural groove* stage containing 3 embryos, *Somite* stage containing 2 embryos, *Limb bud* stage containing 2 embryos.

5.1 General morphology of embryos

5.1.1 Pre-streak stage

The early *pre-streak* stage embryos were spherical blastocysts that had lost the trophectoderm cover of the epiblast. The embryonic disc was seen at one pole of the blastocyst (Figure 5.1 A). In the late *pre-streak* stage the conceptus had changed into a more ovoid form. The same applies to the embryonic disc which also became more oval. A crescent shaped thickening was seen at the posterior end of the embryonic disc. The hypoblast was seen underlying the epiblast and trophectoderm (Figure 5.1 B).

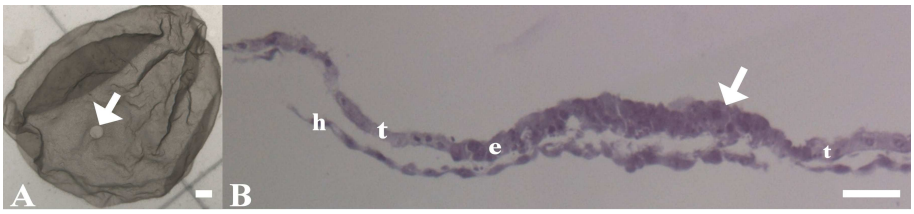

Figure 5.1 A-B: A) The spherical conceptus was seen exposing the embryonic disc (arrow) on the surface at one pole. Bar 500 µm. B) The sectioned pre-streak stage blastocyst showing the epiblast (e) with its caudal thickening (arrow). The epiblast was seen to be continuous with the trophoblast (t). Underlying the epiblast and trophoblast was the hypoblast (h). Bar 100 µm.

5.1.2 Neural groove stage

The embryos in the *neural groove* stage were no longer spherical blastocyst. Instead they had elongated and were of filamentous appearance. The neural plate and the neural groove are outlined on the embryo proper (Figure 5.2). The neural groove was seen as an indention in ectoderm. Underneath the ectoderm was the mesoderm seen. The en-

doderm was only sparsely seen (Figure 5.2 B)

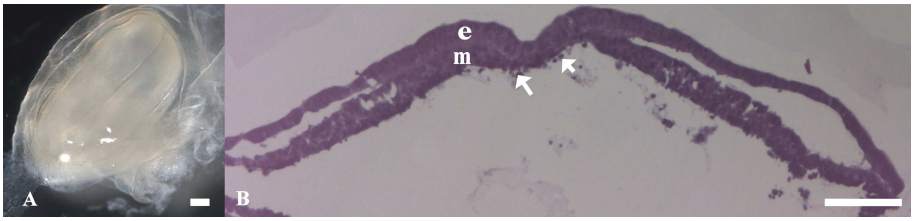

Figure 5.2 A-B: A) Stereomicroscopic photo of neural groove stage embryo. B) Neural groove stage embryo transversely sectioned. The three germ layers are seen with ectoderm (e), mesoderm (m) and sparse endoderm (arrows). The neural groove was seen as the indention in the ectoderm. Bar 100 µm.

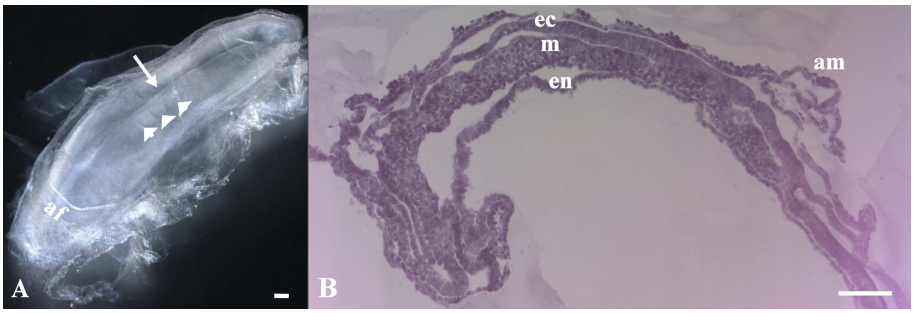

Figure 5.3 A-B: A) Stereomicroscopic photo of somite stage embryo. Somites are seen as condensations of the paraxial mesoderm (arrowheads) lateral to the neural groove (arrow) and the amniotic folds are seen (af). Bar 100 µm. B) Somite stage embryo sagittal sectioned showing the ectoderm (ec), the mesoderm (m) and the endoderm (en). The amnion (am) was seen in the caudal part of the embryo. Bar 100 µm.

5.1.3 Somite stage

At the *somite* stage the paraxial mesoderm had condensed into somites. The neural plate and the neural groove have clearly been outlined and the neural tube started to close. The amnionic folds were seen. The embryo was seen bending downward cranially at the head process (Figure 5.3).

5.1.4 Limb bud stage

At the *limb bud* stage the embryo had started to bud limbs. The optic placode was seen lateral on the head process. The neural tube had closed further. The heart process was seen. The brain ventricles were seen on the sectioned specimen (Figure 5.4).

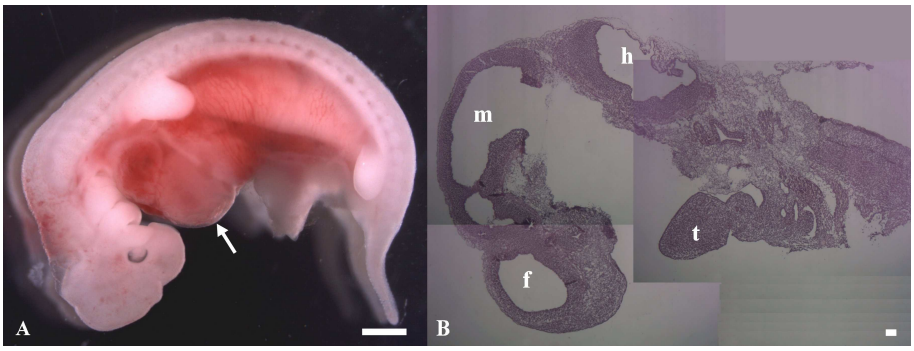

Figure 5.4 A-B: A) Stereomicroscopic photo of *limb bud* stage embryo. The embryo is seen with its head process bending forwards and its limb buds. The heart process (arrow) is seen next to the bending head process. Bar 1 mm. B) Head of *limb bud* stage embryo sagittally sectioned showing the forebrain (f), the midbrain (m) and the hindbrain (h). The tongue anlage (t) is at the entry of the primitive mouth stomodium. Bar 100 µm.

5.2 Immunofluorescent observations

5.2.1 Expression of PAX7

The three embryos included in the *pre-streak* stage were not expressing PAX7. There was no nuclear or cytoplasmatic staining to be seen anywhere in the embryonic disc.

At the *neural groove* stage a transversely cut part of the closing neural groove of one embryo showed expression of PAX7. This expression occurred in anticipated early neural crest cells lateral to the opposing neural folds (Figure 5.5). This expression was concentrated to the nuclei as seen in the insertion of the red filter layer in Figure 5.5 B.

There was not seen any expression of PAX7 in the sagittally cut *somite* stage embryos.

The *limb bud* stage specimens showed a clear expression of PAX7 in the wall of the neural tube, especially in the dorsal one third of the tube (Figure 5.8). The expression appeared confined to the nuclei of the cells. There was also seen an expression of PAX7 in the dermomyotome lateral to the neural tube (Figure 5.8).

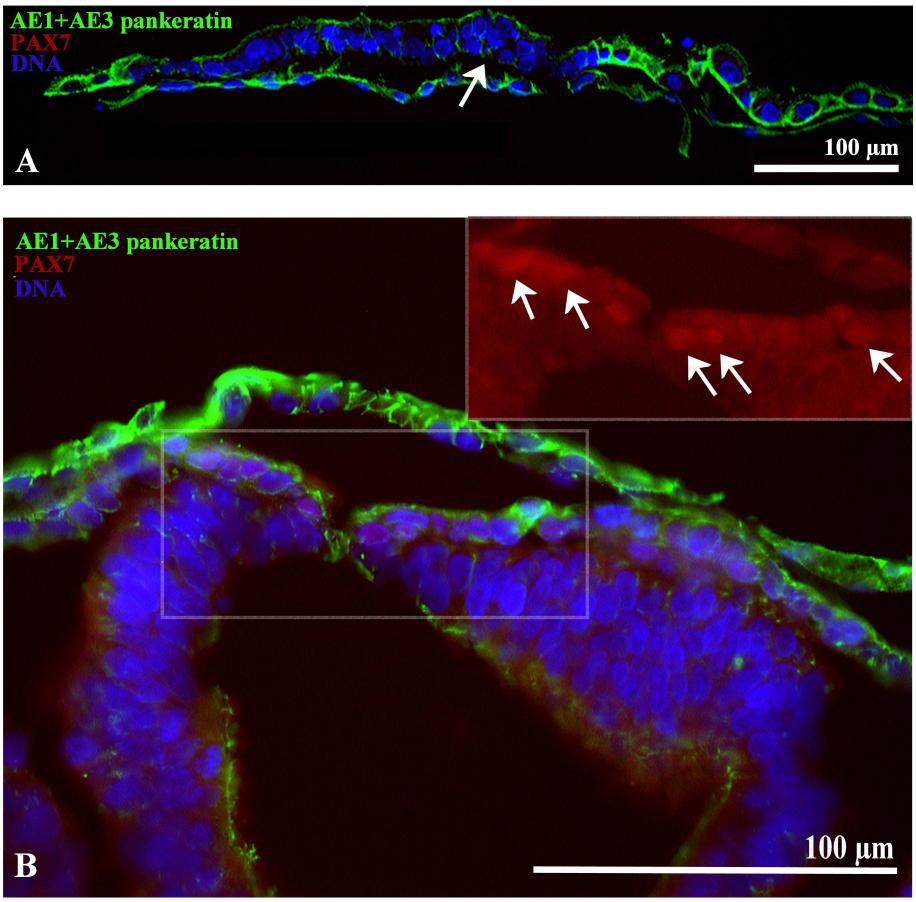

Figure 5.5 A-B The expression of PAX7 and AE1 and AE3 pankeratin was seen at various stages. A) *Pre-streak stage* embryo that showed no expression of PAX7 in the embryonic disc which here was attempted sectioned sagittally. The crescent shaped thickening (arrow) was used as a directional mark. B) The closing neural tube seen in a *neural groove* stage embryo showed expression of PAX7 in nuclei of assumed neural crest cells. Box marks area of insertion. Insertion shows red filter layer from sketched area with nuclei positively stained for PAX7 (arrows).

5.2.2 Expression of SOX2

The three embryos included in the *pre-streak* stage were not expressing SOX2. There was no nuclear nor cytoplasmic staining to be seen anywhere in the embryonic disc. In the *neural groove* stage no expression of SOX2 was seen in nuclei or cytoplasm.

In *somite* stage embryos there was not seen any nuclear expression of SOX2. There was a staining in the cytoplasm of one embryo where no staining could be seen in the nuclei (Figure 5.6). It was seen on both sections on the slide glass. However there was not seen neither cytoplasmic nor nuclear staining on the parallel section stained for SOX2 and NF-protein.

The *limb bud* group showed a clear expression of SOX2 localized to cells in the wall of the neural tube. This expression was seen confined to the nuclei. (Figure 5.7).

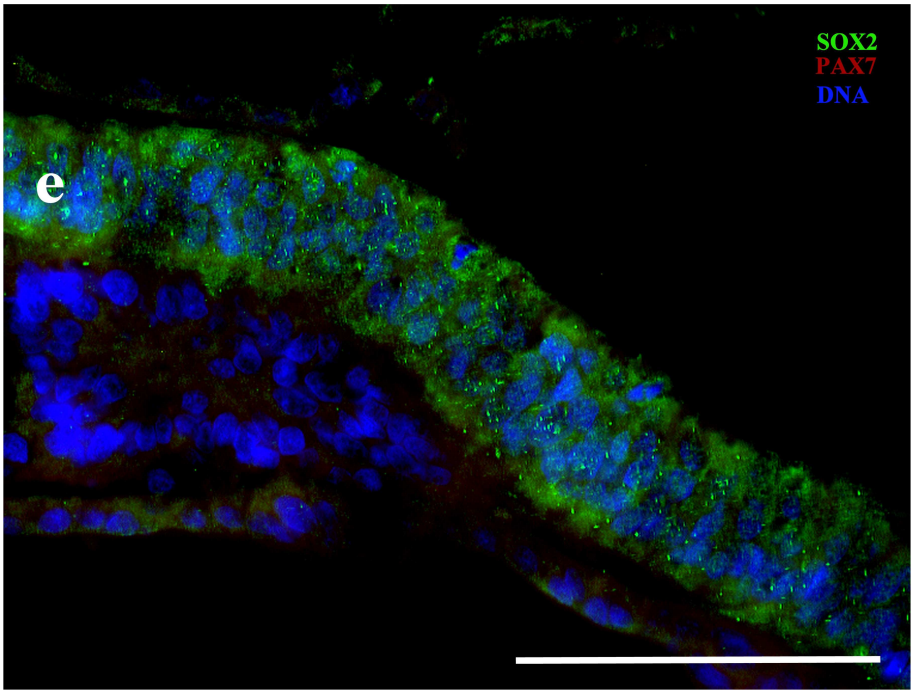

Figure 5.6: Expression of PAX7 and SOX2. There was seen a cytoplasmic marking for SOX2 in the ectoderm (e) in this somite stage embryo while no staining for PAX7 was observed. Bar 100 µm.

5.2.3 Occurrence of AE1 + AE3 pankeratin

The three embryos included in the *pre-streak* stage were all expressing AE1 + AE3 pankeratin. Expression was seen in the epiblast where there was a clear cytoplasmic staining. Besides a cytoplasmic expression was seen in the hypoblast and trophoblast (See Figure 5.5 A).
In the *neural groove* stage all embryos expressed AE1 + AE3 pankeratin in their surface ectodermal cells cytoplasm. Furthermore expressions of AE1 + AE3 pankeratin were seen in endodermal cells as well as in hypoblast cell cytoplasm. A cytoplasmic expression of AE1+ AE3 pankeratin was seen in *somite* stage embryos.
In the *limb bud* stage there was a very clear cytoplasmic expression of AE1+AE3 pankeratins in the surface ectoderm of the head and oral cavity. Furthermore the notochord was expressing pankeratins very distinctly (Figure 5.8).

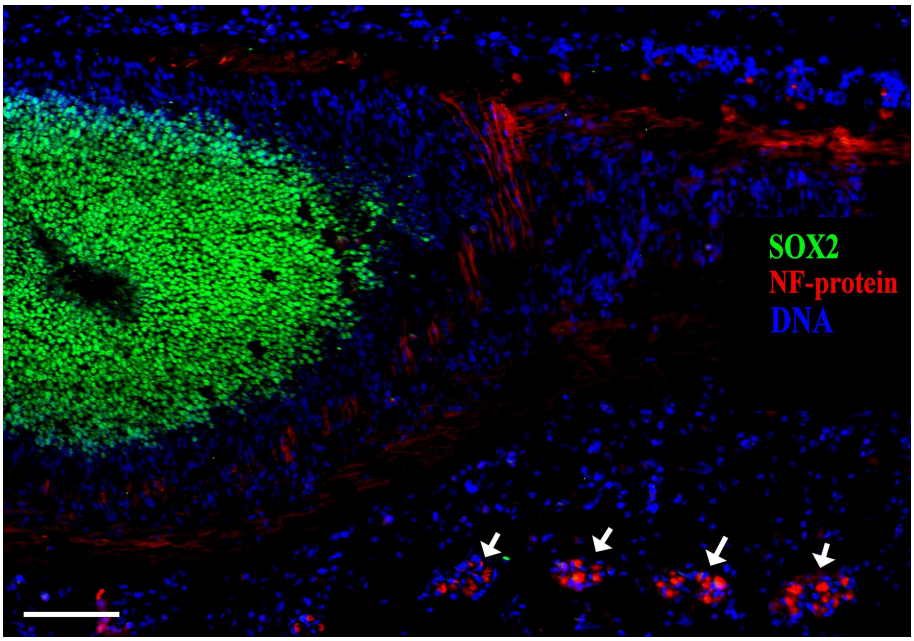

Figure 5.7 The expression of SOX2 and NF-protein in a *limb bud* stage embryo. The neural tube had been transversely sectioned. Occurrence of SOX2 was seen restricted to the nuclei of neural wall cells. NF-protein was seen as filamentous streaks. Transverse cut peripheral nerves wee seen with axons expressing NF-protein (arrows). Bar 100 µm.

5.2.4 Occurrence of NF-protein

Staining for neurofilament protein was not observed anywhere but the *limb bud* stage. In the *limb bud* stage the neurofilaments were seen distinctly as filamentous streaks. The directions of the filamentous streaks went in a longitudinal and a tangential direction as regards to the neural tube. They were also seen in axons in transversely cut peripheral nerves (Figure 5.7).

Table 5.1 Overview table of the occurrence of PAX7, SOX2, AE1+3 pankeratin and NF- protein during the different embryological stages.

Stage	PAX7	SOX2	AE1+3 pankeratin	NF-protein
Pre-streak	-	-	+	-
Neural groove	+	-	+	-
Somite	-	(-)	+	-
Limb bud	+	+	+	+

(-) indicates the occurrence of a tentative positive staining.

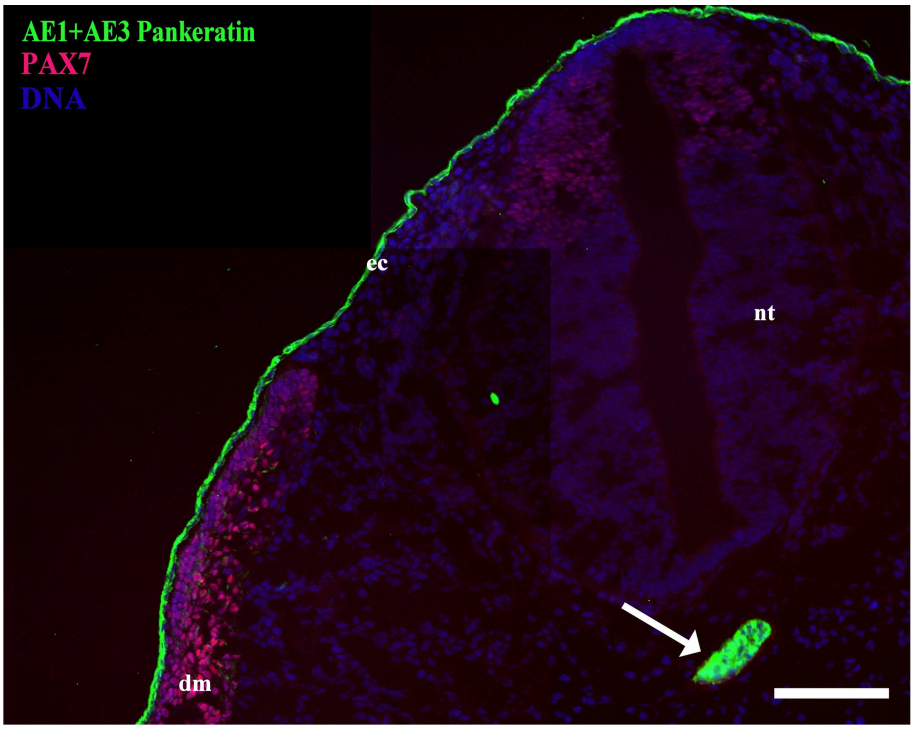

Figure 5.8: The expression of PAX7 and AE1 +AE3 pankeratin in a transversely sectioned *limb bud* stage embryo. Expression of PAX7 was seen in the dorsal one third (arrow) of the neural tube (nt) as well as in the dermomyotome (dm). Expression of AE1+AE3 pankeratin was seen in the surface ectoderm (ec) and in the notochord (arrow). Bar 100 µm.

5.2.5 Negative control

The negative control group consisted of a *somite* stage embryo and a *limb bud* stage embryo. Slides were double incubated with murine IgG2 and stained with goat anti mouse Alexa 594 and 488 in that order. There was seen a weak stain in the surface ectodermal cells with Alexa 488 in the *limb bud* stage embryo when the gain was turned up (Figure 5.9 A). There was not seen any staining on the *somite* stage negative control (Figure 5.9 B).

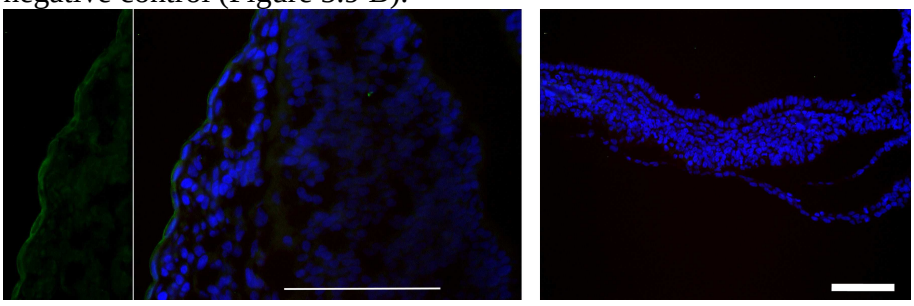

Figure 5.9 A-B: A) *Limb bud* stage negative control with a weak stain of the surface ectodermal cells when camera shutter time value was very high. Insertion shows green filter layer of surface ectoderm. Bar 100 µm. B) Somite stage embryo as negative control. Bar 100 µm.

5.2.6 Co-localization

Seeking co-localized expressed antigens it can be seen from Table 5.1 that the *neural groove* stage expression of PAX7 and AE1+3 pankeratin was the only candidate besides what was seen in the the *limb bud* stage. As seen in Figure 5.5 B the cells that expressed PAX7 in the nuclei also showed expression of AE1 + AE3 pankeratin in their cytoplasm. But this was not a co-localization since it was not expressed in the same cellular compartment.

The *limb bud* stage specimens showed a clear co-localization of PAX7 and SOX2 in the dorsal third of a transverse sectioned neural tube (Figure 5.10). The expression was seen to be in the nuclei. The expression of SOX2 extended to the whole neural tube while PAX7 was restricted to only the dorsal third.

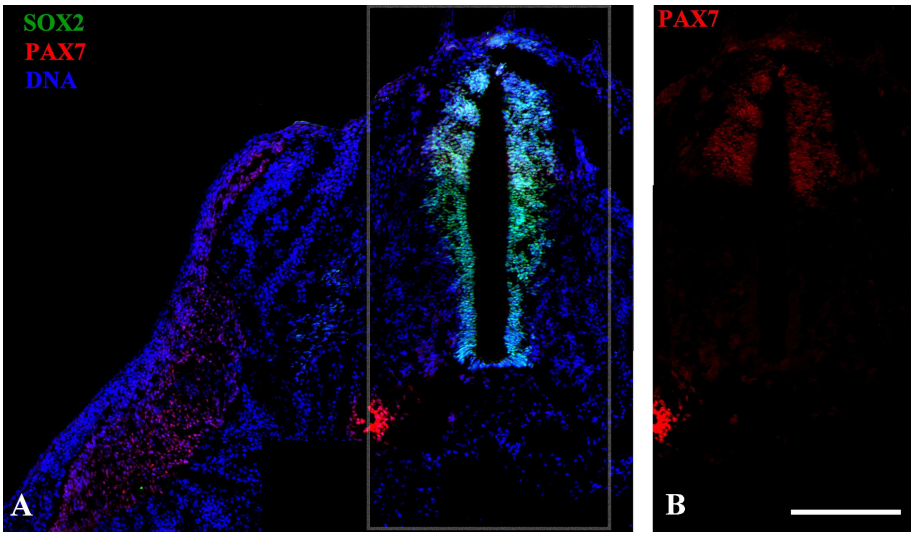

Figure 5.10 A-B: A) Transverse section of the neural tube of a *limb bud* stage embryo. Dorsal one third of neural tube shows intranuclear double staining for PAX7 and SOX2. Ventral two thirds show only intranuclear expression of SOX2. Dermomyotome shows expression of PAX7. The box marks the area of B). B) Red filter layer showing to expression of PAX7 in the dorsal one third of the neural tube. Bar 100 µm.

6 Discussion

The purpose of this study has been to characterize the expression of PAX7, Sox2, AE1+3 pankeratin and neurofilament protein with respect to localization in the early development of the porcine nervous system.

No expression of PAX7 was observed in the *pre-streak* stage. At the *neural groove* stage it was seen confined to the nuclei in a few cells in the transition between the neuroectoderm and the surface ectoderm i.e. the neural crest. This fits well with the fact that PAX7 is a transcription factor. The expression in only few cells in the neural crest cell population shows a very specific stain.
Unfortunately no expression was in the *somite* stage embryos. This might be explained by the fact that none of the somite stage embryos were transversely sectioned. The fact that the somite stage embryos only had been cut sagittally, made it difficult to ensure the presence of neural crest cells. The reason why there was a transverse section of the neural groove embryo was due to downward bending of the embryo which made perfect sagittal alignment difficult.
The occurrence of PAX7 in the neural crest cells seems to fit perfectly with the description of PAX7 in chicken by Basch *et al.* (Basch *et al.*, 2006). The expression of PAX7 in the *limb bud* stage was seen very distinctly in the dorsal one third of the neural tube and in the dermomyotomes. This corresponds well with the literature (Chi and Epstein, 2002). The expression of PAX7 in the dorsal third of the neural tube and in the dermomyotome had been shown in chicken (Lacosta *et al.*, 2005). Lacosta *et al.*, (2005) also showed the expression of Pax7 in non-neural ectoderm cells positioned dorsolateral to the neural tube.

The *pre-streak* stage and *neural groove* stage embryos did not show any expression of SOX2. The *somite* stage expression of SOX2 seen must be considered a false positive for several reasons. For one it was a cytoplasmatic staining not a nuclear. Secondly it was not seen in the parallel section also stained for SOX2. There was no good explanation to the marking besides some error during staining. The difference between the two *somite* stage slides was that the false positive had been incubated with monoclonal mouse anti chicken PAX7 in the first incubation and that is subsequently had been blocked in paraformaldehyde and microwaved. The *somite* stage slide not showing SOX2 had been stained so that monoclonal mouse anti human SOX2 was the first incubation as opposed to the second in the other slide.
The *limb bud* stage showed a clear expression of SOX2 in the nuclei of

the cells in the neural tube.
The sparse expression of SOX2 during the development was in contrast to what was shown by Sundkvist on paraffin sections (Sundkvist, 2006) and what was seen from the *limb bud* stage (Figure 5.8 and Figure 5.10).
There are several differences to consider. The paraffin sections had been prepared immediately after collection and had been stored in paraffin blocks. The cryo sections had been stored at 4 º C in 1 % paraformaldehyde for 1 year. On the paraffin sections there had been used antigen retrieval which means treating the specimens in a microwave oven in a citrate buffer. An equivalent treatment were used on the cryo sections to block to first primary-secondary antigen bindings using the method of Tornehave *et al.*(Tornehave et al., 2000). One should believe that it would work as antigen retrieval as well. This raises the question whether the prolonged storage had been damaging the specimens rendering them to express less or different of the epitopes sought. This suspicion can be further backed up by the perfectly stained control specimens.

All embryos showed expression of AE1+AE3 pankeratin. The expression was seen in the cytoplasm of epiblast and hypoblast and later in ectodermal and endodermal cells. It was also seen in the trophectodermal cells. There was a very distinct staining especially the surface ectoderm. The notochord was also seen to stain for AE1+AE3 pankeratin in the transversely cut control embryo. There was a stain on the negative control (See Figure 5.9A) at the position of the surface ectoderm. As formerly described the camera's exposure time had to be set at a high time value compared to the normal exposure time for a positive stain This suggests that the stain originated from normal tissue autofluorescence.

First at the *limb bud* stage was the expression of neurofilament protein observed. The *limb bud* stage embryos showed a perfect stain for neurofilament protein outlining axons and nerves travelling in different directions in the neural tube. Ventral to the neural tube there were also seen what must be axons in transversely cut nerves (See Figure 5.7). The complete absence of labelling at earlier stages raised the question of whether the quality of the specimens was proper.
Besides it can also be discussed whether it would have been easier to see staining if the embryos were transversely sectioned instead of sagittally.

PAX7 and AE1+AE3 pankeratin were seen expressed in the same cells in the neural crest area in Figure 5.8 B. The neural crest cells originate

from the area between the neural plate and the surface ectoderm. The expressions of surface ectodermal features as well as neural crest transcription factors suggest that the cells could have the ability to differentiate into both. This raises the question whether the cells are precursors for as well neural crest cells as ectodermal cells or simply just premigratory neural crest cells before the onset of migration. The occurrence of cells expressing PAX7 in the same position has already been reported in the chicken where the cells were addressed as non-neural Pax7-positive ectoderm cells (Lacosta *et al.*, 2005). But whether the cells are ectoderm cells or premigratory neural crest cells has yet to be answered.

7 Conclusion

Localization of expression of PAX7 was demonstrated in pig embryos for the first time. Labelling was seen in the neural crest cells in the *neural groove* stage and later it was seen expressed in the dorsal one third of the neural tube and in the dermomyotomes of *limb bud* stage embryos. The expression of PAX7 was co-localized with SOX2 in the dorsal one third of the neural tube in the *limb bud* stage where both are expressed in the nuclei.
The expression of SOX2 was observed in the *limb bud* stage, but there was not observed any labelling for SOX2 within any of the other groups.
The AE1+AE3 pankeratin expression was observed in the cytoplasm of ectodermal and endodermal cells on the embryo proper. The trophoblast was expressing AE1 + AE3 pankeratin as well. The notochord expressed keratins clearly.
PAX7-positive pankeratin-positive cells were observed in the ectoderm just lateral to the neural folds.
Neurofilament protein was observed in the *limb bud* stage. Neurofilament protein was not observed at any prior stages.

8 Perspectives

There are several things that could be done in another manner. One big issue was whether the quality of the embryos used were of a good enough quality. One way to try to answer this question would be to repeat the experiments by taking new embryos use the immunostaining protocol on them without the prolonged storage in paraformaldehyde. This would help to bring light to whether the lack of expression was due to old over-fixed specimens or simply due to lack of expression of the proteins at early embryonic age.
The expression of PAX7 was in the early stages localized to only a few cells. It would seem more obvious to use transversely cut specimens instead of the sagittally cut used for this experiment.
There are several other methods to consider in order to be able to identify the extent of expression. One could use the envision system (DakoCytomation) which is enhancing the signal from the secondary antibodies.
Another method is using *in situ*-hybridization which tells expression on RNA level instead.
Finally it could be very interesting to make a whole mount stain of an embryo and, using a confocal microscope, get a 3 dimensional idea of the extent of e.g. PAX7 expression in the embryo.
While recording the images using the Leica QFish software, making sure that the exposure time is the same for the same filter between different specimens would help to quantify the expression of a specific antigen between different specimens.

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