

Immunohistochemical Investigation of the Proliferation Activity in European Roe Deer (*Capreolus capreolus*) during Embryogenesis after Diapause by Using Ki-67

Miriam Beyes^{1*}, Nelia Nause², Martina Bleyer³, Franz-Josef Kaup³ and Stephan Neumann¹

¹Institute of Veterinary Medicine, University of Göttingen, Germany

²Platform Degenerative Diseases, German Primate Center Göttingen, Germany

³Pathology Unit, German Primate Center Göttingen, Germany

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*For Correspondence

Miriam Beyes, Institute of Veterinary Medicine, University of Göttingen, Burckhardtweg 2, 37077 Göttingen, Germany. Tel: +49-551-3933387
Fax: +49-551-3913919

E-mail: miriam.beyes@gmx.de

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ABSTRACT

The European roe deer (*Capreolus capreolus*) is the most common game in Germany with a high reproduction rate. Moreover, the roe deer has a special reproduction strategy, including an embryonic diapause, which is characterized by a reversible arrest of embryo development at the blastocyst stage. The roe deer embryo is in diapause for about five months. However, there is no complete arrest of embryonic growth, but mitotic activity is reduced to a minimal level. Diapause occurs without causing the embryonic death and after reactivation from diapause, the blastocyst elongates rapidly and embryonic growth is as fast as in other mammalian species. This leads to the conclusion, that embryonic cells after diapause exhibit a high proliferation rate. The Ki-67 protein is widely used as a marker for proliferating cells, because it is present in all active stages of cell division, but it is absent in resting cells. Here we describe the distribution of Ki-67 expression in European roe deer embryos during different stages of development by using immunohistochemistry. In general, the Ki-67 protein is expressed during embryogenesis in roe deer. The immunohistochemical staining of 16 Bouin-fixed and paraffin-embedded embryos revealed, that the Ki-67 protein was detectable in early post-implantation stages of embryonic development. The distribution pattern and staining intensity of Ki-67 expressing cells varied in different organs of the embryo ranging from clear nuclear signals to no nuclear expression of Ki-67 antigen in active stages of cell division. The Ki-67 antigen was almost ubiquitously expressed in the larger embryos with high abundance in the liver. In contrast there were only weak or no staining signals in different organs of smaller embryos.

INTRODUCTION

The European roe deer (*Capreolus capreolus*) benefits from a particular reproduction strategy, because it is the only artiodactyl (*Artiodactyla*) with obligatory embryonic diapause (also known as delayed implantation) ^[1]. The ability to arrest embryonic development occurs in many other orders of mammals, including marsupials (*Marsupialia*), insectivores (*Insectivora*), bats (*Chiroptera*), edentates (*Edentata*), carnivorans (*Carnivora*) and rodents (*Rodentia*) ^[2]. So far, embryonic diapause has been identified in more than 130 mammalian species ^[3]. Embryonic diapause is characterized by a temporary arrest or retardation of embryonic development ^[2] and delays the development of the embryo at the blastocyst stage ^[3-6] resulting in an extension of gestation.

Roe deer has a mono-oestrus and mating takes place in July or August ^[7]. Afterwards embryonic diapause occurs between early August until the end of December or early January ^[8]. The blastocyst loses its zona pellucida after reaching the uterus and during a period of about five months the cell proliferation in the blastocyst is reduced but not absolutely arrested ^[4,8,9]. At the end of December or the beginning of January, a period of rapid embryonic growth follows resulting in placental attachment ^[8,9]. Finally, fawns are born between April and June as their growing up benefits from the mild environmental factors of the spring season ^[10].

Therefore the European roe deer is the most widespread game in Europe with a very high population density^[11,12]. Most fawns are twins and occasionally three fawns are born^[8,13].

For many species, which undergo embryonic diapause (including the European roe deer), the exact duration of the embryonic diapause and the exact point of reuptake are still unknown. Almost nothing is known about the mechanisms by which the maintenance of diapause is organized in roe deer^[4]. It was reported, that the length of embryonic diapause in roe deer is genetically fixed and that the embryo continues development at a certain developmental stage^[14]. In spite of extensive research, the embryonic diapause remains an evolutionary phenomenon whose clarification could implicate general expertise.

The reduction of mitosis in the blastocyst can occur at the G0/G1 or G2 phase of the cell cycle depending on the species^[15]. This anti-proliferative mechanism operates for months without sustainable impairment of the embryo^[16]. Accordingly, apoptosis in the blastocyst is prevented by the maintenance of a basal metabolism^[15], so that a slow proliferation activity occurs in the trophoblast of roe deer blastocysts^[17]. Moreover, during diapause, certain cells must retain their ability of pluri-/multipotency to ensure reactivation of development^[18]. In early January the resumption of embryonic growth resulted in a rapid elongation of the blastocyst^[8].

In this study, we evaluated the proliferation activity in European roe deer embryos by immunohistochemical detection of the DNA-binding protein Ki-67. In general, a high proliferation rate of cells can be expected in embryonic tissue, because at the end of the embryonic period most of the organ systems are established. In this context, investigation of Ki-67 by immunohistochemistry in embryonic tissue is particularly interesting. The Ki-67 antigen is established as an important marker for proliferating cells in the context of diagnostic and prognostic studies related to tumor diseases^[19-26]. The Ki-67-antigen is only present in proliferating cells, but not in resting cells in G0^[27,28]. The highest expression is described in G2M-phase of the cell cycle^[29]. Accordingly, the monoclonal antibody against Ki-67 allows a simple determination of the growth fraction of a given cell population^[27]. Interestingly, the exact physiological function of the Ki-67 antigen is not completely understood so far and focus of many studies^[30,31]. To our knowledge, Ki-67 protein expression data on different stages of embryonic development in European roe deer do not exist. The European roe deer however, is easily accessible for research studies because it is killed legally during a regular hunting process and one can hold of it without further restrictions. In Germany, sampling of embryos is possible until January, when the regular shooting season for roe deer females ends. Above all, favorable conditions are given for sampling roe deer embryos.

The aim of this study was to demonstrate the expression pattern of Ki-67 in different stages of embryonic development with considerable variations in different organs of the embryo.

In general, Ki-67 protein is expressed in early post-implantation roe deer embryos. In order to investigate the general competence of the roe deer tissue for immunohistochemistry (IHC), we applied the SOX9-antibody on tissue sections of a European roe deer embryo.

MATERIALS AND METHODS

Tissue Samples

From the end of December 2015 to January 2016 43 European roe deer (*Capreolus capreolus*) embryos and fetuses were sampled from uteri of female roe deer, which were shot during the regular hunting season in Saxony-Anhalt, Germany. Therefore, no roe deer was killed especially for this study.

The embryos were immediately immersion-fixed in Bouin's solution *in toto* to preserve tissue integrity. Before paraffin-embedding and after washing in 70% ethanol (EtOH), the embryos were measured by using a caliper and a Zeiss binocular to obtain the crown-rump length (CRL). For the two embryos, which were not curved at the time of sampling, the greatest length (GL) was determined.

Tissue Processing and Immunohistochemistry (IHC)

The embryos were fixed in Bouin's solution for 24-36 hours (depending on size) immediately after removal from the uterus. After several washes in 70% ethanol EtOH for at least 2 days, the embryos were embedded in paraffin. During embedding the embryos were positioned to obtain longitudinal sections. 16 embryos were used for immunohistochemical analysis and serially sectioned at 3 µm.

Primary antibodies used in this study included the anti-monoclonal mouse anti-human Ki-67 antigen-antibody (Clone MIB-1, DakoCytomation, M 7240, 46 mg/ml) at a dilution of 1:50 and the polyclonal antibody SOX9 (Millipore, AB 5535, 1 mg/ml) at a dilution of 1:500.

Immunohistochemistry (IHC) for Ki-67 was performed with an automated immunostaining system (Discovery XT, Roche Diagnostics GmbH, Mannheim, Germany) using the SABC (Streptavidin-Biotin-Complex) method and DAB (diaminobenzidine tetrahydrochloride) for signal detection (DAB Map Kit, Roche Diagnostics GmbH, Mannheim, Germany).

One more advanced Bouin-fixed European roe deer fetus (*Capreolus capreolus*) was dissected for immunohistochemical staining. Different organs (heart, liver, kidney, gut and testicle) were embedded in paraffin and sectioned at 3 µm. Gut sections

from this European roe deer fetus served as a positive control for Ki-67 to assure cross reactivity. Negative control stainings were carried out omitting the primary antibody.

Immunohistochemistry (IHC) for SOX9 was performed manually. Tissue sections were deparaffinised, rehydrated, and an antigen retrieval step was performed by microwaving the sections in citrate buffer (10 mM Tri-natriumcitrat-dihydrate in H₂O) for 10 minutes. Endogenous peroxidase was inhibited by incubation with peroxidase blocking reagent (EnVision™ FLEX Mini Kit, High pH, Code K8024 Dako). All incubation steps were performed in a humid chamber and the primary antibody was incubated overnight at 4 °C. EnVision™ FLEX/HRP-kit was employed for signal detection. 3,3'-diaminobenzidine (DAB) chromogen was used as substrate for the HRP and Mayer's hematoxylin was used as counterstain.

Histopathological Examinations

IHC stained sections of 16 representative embryos were scanned with the Aperio CS2 eSlide Scanner from Leica Bio systems. Photos were taken in a lateral view from the left side of the embryo in a 40 times magnification using the Aperio eSlide Manager from Leica (Aperio Image Scope) and in a 63 times magnification using a Zeiss microscope (AxioPHOT) and the Nuance™ multispectral camera.

RESULTS

All embryos were assigned to the post-implantation period. 43 European roe deer embryos were evaluated morphometrically by measuring either the crown-rump length (CRL) or the greatest length (GL). Based on these data, 16 embryos were utilized for immunohistochemical staining, representing different stages of development. These 16 embryos were classified into three main groups (**Table 1**) after histopathological examinations. The size of the 16 embryos ranged from a crown-rump length of about 4.2 mm (respectively from a greatest length of about 6.1 mm) to a crown-rump length of about 27.2 mm (**Table 1**). Pictures of three of these embryos (one embryo of each group) are shown exemplarily in this study. Details of immunohistochemical analysis are described in the following paragraphs.

Table 1. European roe deer embryos used for immunohistochemical staining. CRL: Crown Rump Length; *GL: Greatest Length

Embryo (Serial Number)	CRL [mm]	Group Number
1	6.1*	I
2	6.3*	
3	4.2	
4	5.6	
5	5.9	
6	6.7	
7	6.1	
8	7.1	
9	8.1	
10	8.6	
11	9.3	II
12	11.4	
13	12.7	
14	17.8	
15	21.7	III
16	27.2	

Ki-67 Expression in the Smallest Embryos

Almost all embryos of this group (n=10) were still curved (except two embryos with the serial number 1 and 2, which were not curved at the time of sampling), and primordia of heart, liver and kidney were clearly distinguishable. They had a crown-rump length between 4.3 and 8.6 mm. Only a few cells were positive for Ki-67 in the smallest embryos. **Figure 1** shows a European roe deer embryo with a crown-rump length of about 4.2 mm, which represents the specimens of group I. A general overview of the location of the organs in the embryo is given in **Figure 2A**. Ki-67 staining signals were undetectable in the liver (**Figure 1B**) and in the heart (**Figure 1D**) of the smallest embryo. Only little Ki-67 expression was observed in the kidney and was mainly located in cells around the epithelium of the tubuli (**Figure 1C**). Only a few mitotic cells were detected without a Ki-67 staining signal. Clear and numerous Ki-67 staining signals were localized in the developing brain system next to the ventricular lumen (**Figure 1E**) and in the otic vesicle (**Figure 1F**). Another mitotic cell with a Ki-67 staining signal was observed in the mesenchyme (**Figure 1E**). In all cases, Ki-67 positive cells were identified as mitotic cells.

Summarizing these data, there was only little or no Ki-67 expression in different organs of the smallest embryos examined. All specimens of group I exhibited this distribution pattern. In fact, on the basis of semi quantitative assessment of slides, a trend towards an increase of Ki-67 expression with increasing embryonic size within the group was noted. The three smallest embryos of group I revealed more mitotic cells with a positive Ki-67 signal than mitotic cells without a staining signal.

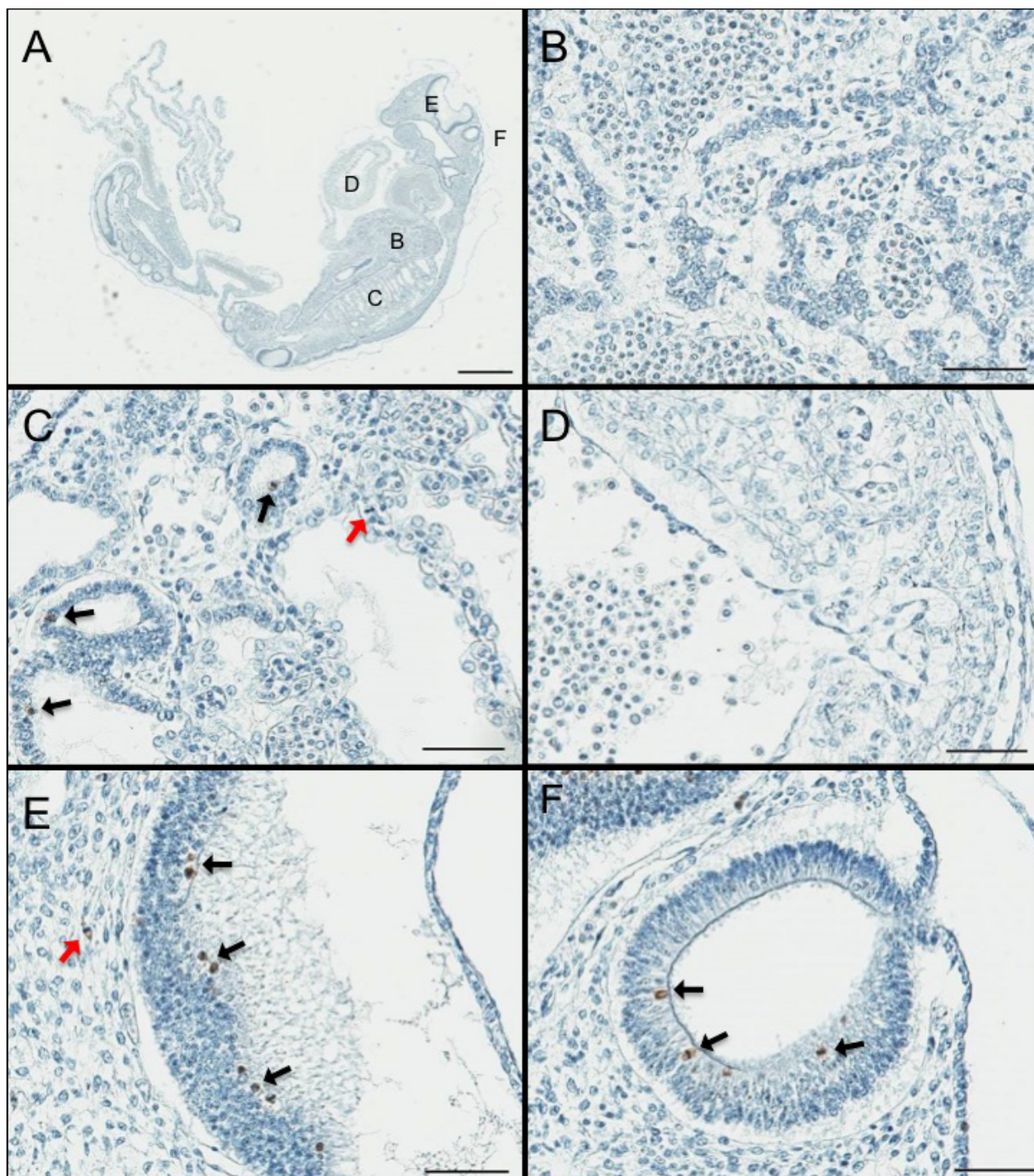


Figure 1. **A** General overview of the Ki-67 expression in a roe deer embryo with a CRL of ~ 4.2 mm. Scale bar=0.5 mm. **B-F** Higher magnification of the roe deer embryo showing Ki-67 expression in different organs. Scale bar=50 μ m.

Ki-67 Expression in the Medium-sized Embryos

These embryos (n=7) are already curved, and the most prominent organ primordia are the liver and the heart (Tab. 1). They had a crown-rump length between 9.3 and 17.8 mm. **Figure 3** shows a roe deer embryo with a crown-rump length of about 9.3 mm, representing the specimens of group II. In comparison with other organs, the liver exhibited the vast majority extensive of Ki-67 staining signals (**Figure 2B**). Interestingly, we also detected a number of mitotic cells with no staining signal in the liver. **Figure 2C** shows a higher magnification of the liver with a metaphase plate and a distinct spindle apparatus, lacking a Ki-67 staining signal. No Ki-67 staining was detectable in the kidney of the medium-sized embryo (**Figure 2D**). Also there is a number of mitotic cells lacking a staining signal. The Ki-67 antigen was undetectable in the heart (**Figure 2E**). Ki-67 expression was also observed in the eye primordium (**Figure 2F**) as well mitotic cells in the developing lens and in the inner layer of the retina. Only the embryo with a crown-rump-length of about 11.4 mm exhibited a number of metaphase plate lacking Ki-67 staining signals.

In summary, Ki-67 expression was moderately expressed in all medium-sized embryos. In addition to cells with Ki-67 staining, numerous mitotic cells without a Ki-67 staining signal were observed in this group.

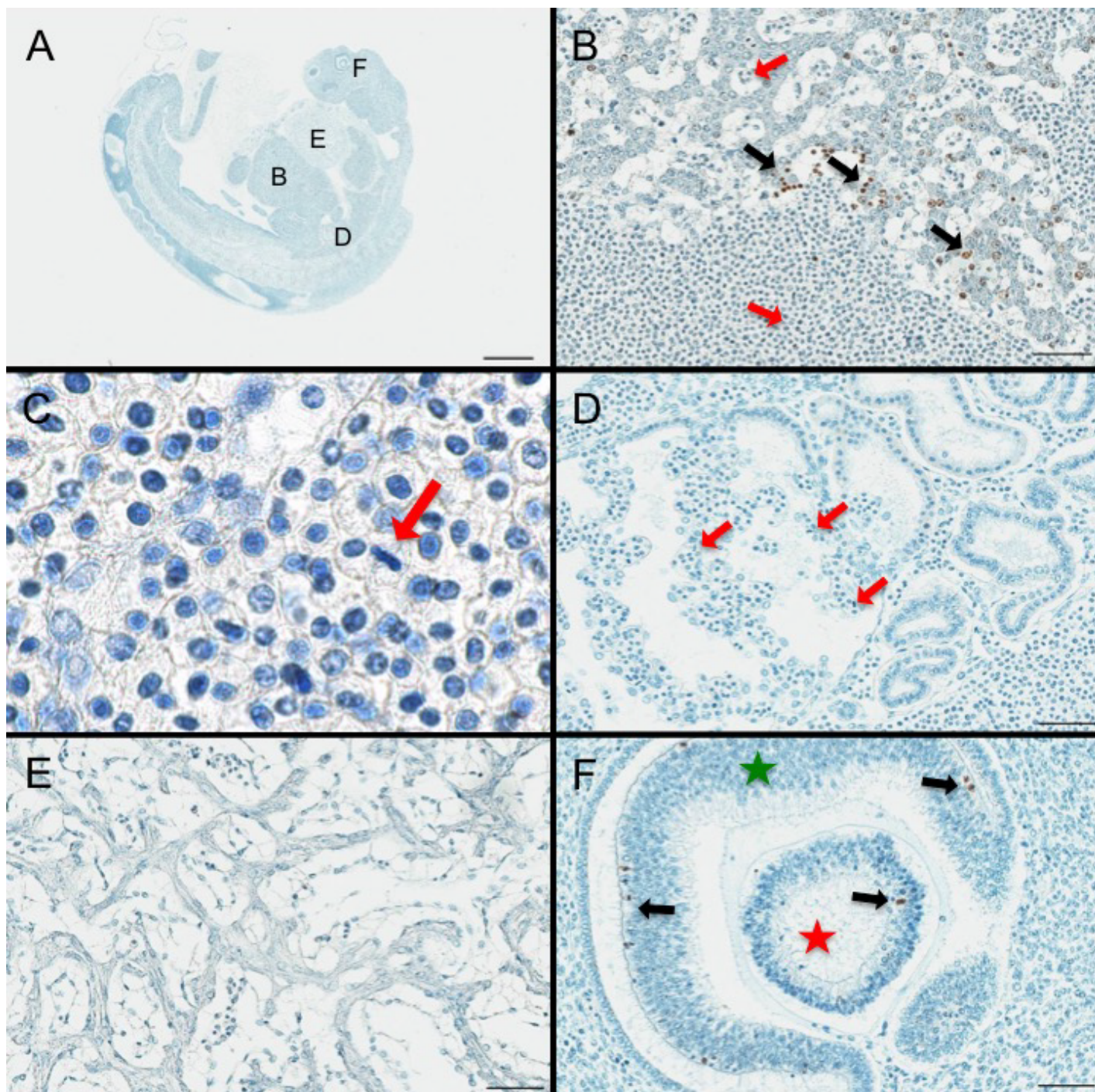


Figure 2. A General overview of the Ki-67 expression in a roe deer embryo. CRL of the embryo is ~ 9.3 mm. The scale bar represents 1 mm. **B - F** Higher magnification of the roe deer embryo showing Ki-67 expression in different organs. Scale bar=50 μ m.

Ki-67 Expression in the Largest Embryos

Two embryos of this group presented a crown-rump length of about 27.2 respectively 27.2 mm (**Table 1**). Both embryos showed an almost identical distribution pattern of Ki-67 expression. The staining signal was widely distributed throughout the embryos (**Figure 3A**). The most prominent developing organ in the embryos was the liver, where Ki-67 expression was highly abundant and located in the nuclei of many cells (**Figure 3B**). There were also prominent Ki-67 signals in the kidneys (**Figure 3C**) and in the lung (**Figure 3E**). In the kidney, staining signals were mainly located in the tubular epithelium. In contrast to other organs, fewer signals were detected in the heart (**Figure 3D**). Numerous staining signals were observed in the head of the embryo, with highest density in the developing brain system (**Figure 3F**). Ki-67 signals were in close association to the ventricular lumen and in the choroid plexus. No obviously mitotic cell lacking a Ki-67 staining signal was identified in these embryos.

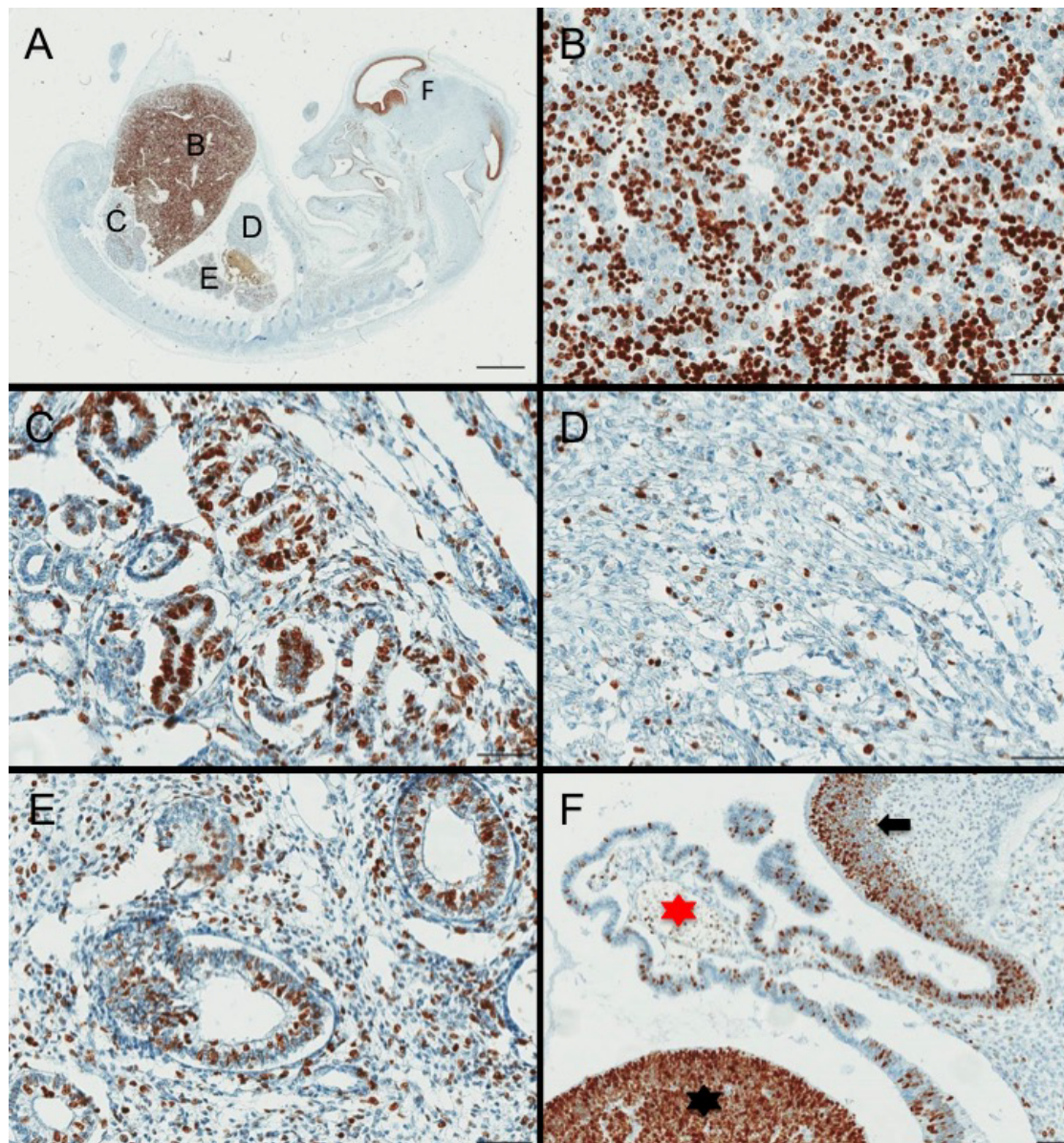


Figure 3. A General overview of the Ki-67 expression in a roe deer embryo with a CRL of ~ 27.2 mm. The scale bar represents 2 mm. B-F Higher magnification of the roe deer embryo showing Ki-67 expression in different organs. Scale bar=50 μ m.

DISCUSSION

According to a general assumption, embryos are characterized by a very high proliferation rate. The European roe deer embryo (*Capreolus capreolus*) has the particular ability to arrest embryonic development reversibly over a period of several months with a minimum of mitotic activity [4,17,32]. Finally, termination of embryonic diapause starts with the resumption of mitotic activity [15] and in the middle of December embryonic growth is as fast as in other species without embryonic diapause [33]. In this context we investigated the proliferation activity of embryonic cells in roe deer by using immunohistochemical staining for Ki-67 antigen. Furthermore, there is a relationship between the Ki-67 antigen and cells of the growth fraction [34] as well as the Ki-67 protein expression is correlated with the physiological state of the cell [35]. Moreover, in a recent study, proliferation in roe deer testicles was detected by immunolocalization of Ki-67 [36].

To our knowledge, the present study describes the Ki-67 antigen expression during embryonic development in European roe deer for the first time and revealed an interesting Ki-67 distribution pattern. As a general result of our examination, we observed nuclear Ki-67 expression in every examined stage of embryonic development and could show that Ki-67 is expressed early in post-implantation embryos analyzed so far. Immunohistochemical examinations exhibited that Ki-67 expression depended on the crown-rump-length of the embryo, in that it increased with increasing embryonic size. Accordingly, we confirmed strong nuclear Ki-67 expression in the larger embryos utilized in this study, where the Ki-67 antigen was almost ubiquitously expressed. Strong Ki-67 expression was also detectable in the European roe deer fetus, which was analyzed for verification of the specificity and cross-reactivity of the Ki-67 antibody. However, a different Ki-67 distribution pattern with distinctly weaker staining signals was

detected in the smaller embryos of this study. These data gave reason to further explore the general competence of the roe deer tissue for immunohistochemistry in smaller embryos. Therefore, we applied the SOX9-antibody on tissue sections of a European roe deer embryo (*Capreolus capreolus*), because SOX9 was already taken into account in a study about intersex in roe deer [37]. The results confirmed that the smaller embryos still have the competence for immunohistochemistry.

The Ki-67 protein expression in different organs of the embryos was highly variable. There was no consequent distribution pattern in the respective organs in different stages of development. While in some embryos, the hepatic tissue exhibited the highest amount of Ki-67 staining signals, it was the kidney in other embryos. In this context, one could assume that the different distribution patterns in various embryos are associated with certain stages of development of the particular organ during embryogenesis. On the other hand, we observed organs omitting a Ki-67 staining signal but exhibiting mitotic figures. Also, we observed roe deer embryos with numerous mitotic figures lacking a Ki-67 staining signal. Moreover, the number of Ki-67 negative mitotic cells was dependent on the crown-rump-length of the embryo. Embryos of a crown-rump-length between 5.6 mm and 11.4 mm revealed an obviously higher number of mitotic cells lacking a Ki-67 staining signal compared to the smallest embryos with a crown-rump-length of less than 5.6 mm and the larger embryos with a crown-rump length of more than 11.4 mm.

Little is known about the physiological function of Ki-67 during cell division. Ki-67 is strictly associated with cell proliferation [38] and, so far, a cell division-controlling function for the Ki-67 protein has been assumed [30]. Flow cytometric analyses have shown that the Ki-67 signal has its highest staining intensity in mitotic cells [39]. An intense perichromosomal Ki-67 staining signal has its peak in early mitotic cells, whereas the Ki-67 antigen decreases rapidly at the end of the M-phase [30]. However, mitotic activity can take place without the presence of Ki-67, at least in embryonic tissue as it could also be shown in the present study. Therefore, it can be assumed that the Ki-67 antigen is not essential for cellular proliferation. Alternatively, another protein inherits the function of Ki-67 during cell division in embryonic tissue. Because of the presence of Ki-67 negative and positive mitotic figures in the same embryo, it could be possible that another protein operates that has the same function as Ki-67. Indeed, the number of Ki-67 negative mitotic cells differs widely within the embryos observed. This raises the question whether the antigen Ki-67 is continuously expressed during embryonic development or not.

Further investigations are needed to clarify whether the Ki-67 protein is already detectable in the blastocyst stage. In many mammalian species, the embryonic diapause starts at the blastocyst stage of embryonic development [4]. In roe deer, the hatched blastocyst is in proliferation arrest for about five months and suddenly increases rapidly before implantation [8]. The obvious reduction of mitotic activity does not lead to death of the embryo [16]. This exceptional ability makes the blastocyst a further interesting stage of development for experimental research, especially for reproductive technologies on animal breeding.

The presented data demonstrate the general validity of the anti-Ki-67 antibody for immunohistochemical examinations in European roe deer embryos, although Ki-67 was not detectable in every mitotic figure identifiable. To assess these findings in more detail, further roe deer embryos of different sizes should be analysed. In addition, a second marker for proliferating cells should be included to compare staining results, e.g., the proliferating cell nuclear antigen (PCNA), which is associated with the cell cycle [40,41] and has already used in European roe deer [36]. Further examinations are needed to better understand Ki-67 expression during different phases of development in European roe deer embryos, especially in early pre- and post- implantation stages of development.

CONCLUSIONS

In larger post-implantation European roe deer embryos, Ki-67 immunohistochemistry produces a clear and intense staining signal, which seems to be suitable for quantitative analyses. Against our assumption, Ki-67 expression is not ubiquitously present in all stages of embryonic development and decreases in embryos of smaller size or of an earlier stage of development, respectively. The detection of Ki-67 negative cells in mitotic stages gives reason for further investigations.

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