1. Title of project
Heart development in primates

2. Purpose of animal test and report of past results, if any
Congenital malformations of the heart are the most common fetal malformation in children. Some of the malformations are uncomplicated and need no treatment while other congenital heart diseases are life threatening and need treatment immediately after birth. Therefore, it is of great medical relevance to try understanding how the heart develops and what goes wrong in the development process of congenial heart disease. It is well documented that genetic factors are of great importance in the development of the disease with congenital malformations of the heart, and research in many laboratories around the world, including my own research group, have identified genes that are necessary for a normal development of the hearts of fetuses.

We have earlier discovered that the majority of the heart tissue as well as basically all myocardial cells are developed from two cardiac-specific stem cell populations. In earlier tests, performed in my USA-based laboratory, we have produced various genetically modified mice and have been able to characterize their cardiac stem cells, which genetic programs that control their biology, and which parts of the heart they originate. Based on this information we have an idea of which specific genes could be essential to the cardiac development. Unfortunately, results from tests on mice cannot be directly adapted to humans. Earlier, we have proved that there are basic differences between the ways cardiac stem cells build up the mouse heart and how the human heart is structured based on size, complexity and the length of cardiac development, spanning from a few days in mice to months for humans. There are also great differences between the physiology and heart rate in mice and humans. In the matter of heart malformation, which arise during the fetal development and are an important cause for heart disease in infants, it is important to use animals which are as close to humans as possible biologically. In addition, we hope to determine if the severe forms of malformations of the heart in children are caused by a primary defect in these particular stem cells, and we believe that it is highly probable that a subset of congenital heart defects in children is a disease of the stem cells. In this case, the progress in stem cell biology would enable new therapeutic strategies with infants born with life-threatening disease.

The purpose of the experiments covered by this application is to study the embryonic heart development in primate embryos. For this, we want to use normal embryos as well as primate embryos that have gen modifications which cause congenital heart-diseases. Earlier, we have established new effective techniques of causing specific gene mutations in mouse-models. These techniques have also been used with primates by other groups but for other purposes. By finding the specific problems in cardiac development which arise in animals with gene modifications, we will gain important information for trying to prevent these problems and in that way develop strategies of treatment for prenatal heart malfunctions. All the experiments are conducted at Astrid Fagraeus Laboratories which has advanced experience in primates as test animals and conduct their business according to the highest ethical standards. Furthermore, through our cooperative relations with nearby Karolinska Sjukhuset, we can also use the expertise of these specialists to assure the highest quality of the implementation of the experiment.

3. Other methods than the chosen
The experiments covered by this application are intended to identify the genes which cause important cardiac malformations during the fetal period. We have, through experiments in mouse models and cell-culture systems and literature studies identified important candidate genes which are essential to the normal development of the heart. There are, unfortunately, too many differences between the development of mouse and human hearts; results from research trials in mice cannot be directly adapted to humans. In the matter of heart malformations, which occur during the fetal period and are an essential cause for illness in infants, it is necessary to use laboratory animals who

are biologically as close to humans as possible. Genetically modified apes are the only model system there is to clarify how the heart develops in primates during the fetal period. Therefore, we cannot avoid animal experiments in apes.

5. Choice of animal species, breed and line

The development of the heart during the fetal period is different in different animal species, and the development in rodents, rabbits and other common laboratory animal species are completely different compared to the one in humans. Therefore, it is important to use an animal model where we know that the differentiating stem cells (progenitor cells) that are active during the fetal development develop in the same way as in humans. Basic studies of these cells have, since they were discovered by us 10 years ago, been done on mice and in vitro. In order to move forward with clinical applications on humans, among other things treatment strategies for severe congenital heart defects, studies in apes are necessary. Long-tailed macaques and rhesus apes are the breeds of primates most commonly used in studies of heart development.

6. Trial's time- and implementation plan

In order to control the embryo development and to, in a later stage, implant genetically modified embryos, an in vitro fertilization, IVF, is executed and that cell division has occurred. This takes place with one of our collaborators and frozen embryos are transported to us for further studies. The heart's development in different phases of the fetal development is studied by one or two embryos being implanted in a foster mother's uterus and the fetuses being removed during caesarian operation at given time. The fetus development is followed, after implantation, with ultrasound, and at a given time early in the pregnancy, the fetus is removed with a caesarian for thorough studies of the heart's various cell fractions.

1. Synchronization of time for implantation. A foster mother should menstruate regularly to favor screening for synchronization. Blood samples (abt. 100 ui) are taken daily from day 8 in the menstruation cycle and 5-7 days forward in order to measure estradiol and progesterone. After habituation and training this can be done without the use of anesthetics, but initially a small ketamine sedation may be needed. Even other methods, without blood sampling, for instant saliva, urine or vaginal secretions will be tested.

2a. Embryo transfer using laparoscopy. The ape is tranquilized with ketamine, possibly combined with xylazin or medetomidin. In need of longer narcosis, the ape is put under inhalation anesthesia using isofluran or sevofluran. Complete aseptic is strived for during surgery.

The laparoscopy instruments are inserted through three centimeter-long incisions on both sides of the abdominal midline. The fallopian tube on the ovulating side is located and one or more embryos are inserted 1-3 centimeters into the oviduct. The instruments are removed and the incisions are stitched with 1-2 sutures. Postoperative pain relief (carprofen or similar) is administrated.

2b. Transmyometral embryo transfer. A, for the ape, less invasive method will be tested. This method is successfully used on humans but have yet to be tried on apes. The ape is sedated according to 2a. An ultrasound probe with an attached needle holder is inserted into vagina and a cannula penetrates the vaginal bottom, so that the tip lies in the cavity of the uterus, where one or several embryos are placed. Postoperative pain relief is not needed.

2c. Transabdominal embryo transfer. An alternative to 2b is to, during ultrasound supervision, insert a cannula through the abdominal wall, via the bladder to the uterus cavity in order to attach embryos there. The bladder will need to be filled with saline using a catheter which is inserted in the urethra. This is done to avoid penetration of the bowels with the cannula. The whole procedure is done with anesthetics according to 2a. Postoperative pain relief is not needed.

3.Monitoring pregnancy with ultrasound. From the 3:rd pregnancy week the fetuses can be seen with ultrasound. The ape is lightly sedated with ketamine and the ultrasound probe is pressed to the

abdomen where the hair has been removed and a special gel has been applied. The procedure takes around 10 minutes and is repeated 1-2 times a week until the removal of the fetuses. Training of the apes using positive reinforcement will be done, but if they can't be properly examined, they will be sedated.
4. Caesarian for removal of fetuses. During pregnancy weeks 5-10 when the critical development of the heart's various parts is expected to occur, the fetuses will be removed by caesarian (normal pregnancy is 23-24 weeks). The ape is sedated and prepared according to 2a. The abdomen is opened with a 5 centimeter long incision along the midline close to the pelvic bone. The uterus is located and arranged in surgical cloth. A small incision is placed across the uterus and the fetus/fetuses are removed along with the fetal bladder, the umbilical cord and the placenta. In this stage of fetal development, the fetuses are incapable of breathing and die of asphyxia. Euthanasia and bleeding will still be done through decapitation and the heart is dissected for further studies. The uterus and the layers of the abdominal muscles are stitched with resorbable sutures and the skin with sutures or staples which are to be removed after 10 days, alternatively with intracutaneous (invisible) sutures which will be resorbed. Postoperative pain relief is administered by carprofen or similar.

Normally, an ape can undergo up to four procedures as stated above, with a period of at least 6 months between caesarian operations, after which the ape can be used as an egg donator or, after veterinarian approval, transferred to another trial of lower difficulty.

7. Care and keeping
The animals are kept at Astrid Fagraeus Laboratories by the Karolinska Institute in accordance to the rules and regulations from the Agricultural department and with the fulfillment of all terms issued for keeping animals. The animals are kept two or more in group cages, with at least 2 square meters per ape. The largest cages have a total volume of approx. 50 square meters and holds up to 7 or 10 apes. All apes have daylight in their cages and, if the weather allows, access to the outdoors. During reoccurring samplings, it can however be an advantage for both the animals and staff if the apes are temporarily housed in a cage system with a movable breech and a hatch for taking out a leg for sampling. This cage system can also be suitable for using if certain apes need separating from the group due to incompatibility between various individuals. The animals are trained to cooperate during samplings and such. The animals' environment is structurally enriched with special focus on satisfying the animals' physiological and behavioral needs. Enrichment and training programs for the animals as well as workarounds during social regroupings are developed by our ethologist.

If nothing unforeseen occurs the apes will be able to return to their group right after the various procedures. In the events of complication such as infection or similar, the ape might need isolating for treatment for a few days.

8. The animals' situation and the trial's endpoint
Blood samplings for determination of hormone levels can probably be done without anesthetics as very small amounts are needed. The procedure is quick and shouldn't be stressful for the apes. Should we still experience stress in the apes, a low dose of ketamine will be given. Embryo transfer, ultrasounds and caesarians are done during full narcosis, and postoperative pain relief is given in adequate amounts to prevent any discomfort.
The fetuses will not feel any pain or discomfort as they are removed in a very early stage of development. The procedures done on the apes are to be regarded as mild to moderate difficulty. Whereas the caesarians, if they are repeated, are done with at least 6 months apart, it cannot be seen as reason to increase the severity to considerable. Damages and illnesses can occur in keeping of primates and these are treated when they occur according to veterinarian praxis. Veterinarian is

always at hand to make an assessment of the health of the individual animal, and to apply an adequate treatment.

9. Methods of anesthesia and euthanasia

For blood sampling and ultrasound; Ketamine in small doses (5-10 mg/kg) or normal dose (10-15 mg/kg) IM. For embryo transfer and caesarian: Ketamine (10 mg/kg) and xylazin (0,5 mg/kg) or medetomidin (0,05 mg/kg) IM, or inhalation narcosis with isofluran or sevofluran after ketamine induction (first choice for caesarian).
Post-operative pain relief: Carprofen 4 mg/kg IM. Administered in assessment with surgery and when necessary once each day for 2-3 days. Alternative narcosis and pain relief can be administered under veterinarian supervision.
Euthanasia: Euthanasia fluid while under deep ketamine sedation.

10. Exceptions

(from chapter 10 § 8 “primates may not be used in trials”)
In the case of cardiac malformations that occur during the fetal development and which are an important cause to illnesses in infants, it is necessary to use laboratory animals biologically as close to humans as possible. Apes are the only model system available for clarifying the development of the heart during the fetal period in primates (including humans). Therefore, we cannot avoid animal trials in apes.

11. Summary

Här har jag hoppat över den populärvetenskapliga sammanfattningen då den bara är en förenklad version av allt det som redan beskrivits. Det framkommer ingenting nytt i den.

Appendix

1. Replace

Detta stycke upprepar varför man valt att använda apor istället för möss

2. Reduce

A vast knowledge bank has been built up through previous studies in cell cultures and in mouse model systems. The trial is planned with basis in this experience and we can therefore, in an early stage of the project, use embryos with the exact genetic defects we expect would cause malformations. By taking the fetuses at such an early stage, we can implant several embryos in one ape and thus keep the number of animals used down. The same ape can go through up to four trials and this also contributes to using as few animals as possible.

3. Refine

Basic studies of progenitor cells have, since they were discovered 10 years ago, been made in mice and in cell cultures. To move forward with clinical applications on humans, for instance treatment strategies for congenital heart malfunctions, studies in apes are required. Long-tailed macaques and rhesus apes are the primate species most frequently used in studies of heart development.