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# Spiral Arteriole Remodeling In Rat Deep Placenta Bed: The Progression Changes Of The Early, Trophoblast-Independent Vessel Remodeling.

by

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This paper was prepared

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Professor Sarah J. Bacon

for eight credits.

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# ABSTRACT

In early pregnancy, maternal uterine vessels carrying blood to the placenta are modified to support the development of the placenta. Vessels expand as their walls become disorganized. Trophoblast replaces the original vessel wall. To better understand vessel remodeling, we analyze the changes of the cell type in the vessel wall and categorize those changes. The rats' uterine vessel remodeling stages of histology sections are defined as follows. (1) Unremodeled vessels have organized endothelium and thick media (>10 um). (2) In early remodeling, the media thinned (5-10 um) and endothelium swollen and protruded into the vessel lumen. (3) The endothelium was monolayer, the media layer was 1-5um or discontinuous, and fibrinoid appeared around the vessel's periphery. (4) Trophoblasts were replacing the smooth muscle. The average vessel diameter increased throughout the stages with large variability. The immunohistochemistry section has one more stage with trophoblast fully around the vessel and media starts to break down at stage 2. In conclusion, arterioles smooth muscle and endothelium start to remodel at the same time, well before embryonic tissue (trophoblast) is present. This study helps us understand the sequence of changes during remodeling. Future studies will investigate the maternal factors acting in this remodeling.

# INTRODUCTION

Everyone has a placenta, but it has been processed as biomedical waste right after delivery. Yet the placenta was the baby's lifeline in pregnancy. The placenta is an organ that is both very important and poorly understood.

The placenta is important in a biomedical context, as defects in its development can relate to all kinds of pregnancy disorders, especially preeclampsia, a pregnancy complication characterized by high blood pressure that affects 2% to 8% of pregnant people worldwide and contribute to maternal and fetal morbidity and mortality, especially in people over 35 years. (Tyas, 2020) and people of color (Johnson, 2020). Preeclampsia is due to a defect in the development of the placenta's attachment site in the uterus (Phipps, 2016) and can lead to some pregnancy complications for both mother and fetus such as high blood pressure (Rana, 2019) and fetal growth restriction (Backes, 2011). In this study, we look in detail at the normal development of the placenta’s attachment site in the uterus of the rat. In particular, we will define in detail the progression of changes in the fine morphology of uterine blood vessels that makes successful pregnancy possible.

## General Placenta

The placenta is the first organ to develop in pregnancy, and it develops from a set of tissues outside the embryo proper called “extraembryonic” membranes. It has a crucial set of roles.

The placenta’s main role is to provide oxygen and nutrition to the fetus, especially the fetus's brain, and to eliminate waste products (Maltepe, 2015). To achieve these functions, the placenta has a large surface area for exchange and a thin outer membrane that separates the maternal and fetal circulation. Placenta also remodels the maternal uterine blood vessel which increases the maternal blood flow. Furthermore, the placenta secretes hormones that can influence the maternal metabolism through building up the pregnant people’s energy reserves and releasing these to support fetal growth (Burton,
2015).

In primates and rodents, the placenta brings fetal tissue into direct contact with maternal blood. This is a hemochorial placenta, in which the maternal blood is directly in contact with the fetal extraembryonic membranes. An epithelial cell type called trophoblast forms the outermost layer of the fetal extraembryonic membranes, and it is these cells that bathe in parental blood. To invade maternal tissues, some trophoblasts migrate into the maternal uterine tissue shortly after implantation. Once there, it homes in on specially prepared uterine blood vessels,

taps them, and enables maternal blood to circulate in the placental tissue. In this way, maternal blood is brought into direct contact with trophoblast tissue in the developing placenta (Soares, 2018).

## How Does the Human Placenta Develop

Better understanding the process by which fetal tissue taps into uterine blood vessels requires that we step back a little bit and look at how the tissues of the early embryo form. When blastocyst forms in the first trimester (Day 4-5 after fertilization), it has two layers: an outer layer called trophectoderm (a layer of trophoblast cells) and an inner cell mass that forms the embryo (Figure 1). During attachment and implantation (Day 6), the trophectoderm adjacent to the inner cell mass interacts with and then penetrates epithelial cells lining the uterine lumen.

Around gestation day 8 in humans (Figure 1), the trophoblast cells differentiate into two categories: cytotrophoblast and syncytiotrophoblast. The syncytiotrophoblast, shown in light red in Figure 1, is on the outer layer of the embryo and initially invades the uterus (Knöfler, 2019). The cytotrophoblast, shown in darker red in Figure 1, can differentiate into highly invasive cell types that reach deep into the uterus, all the way to the parent's uterine blood vessels.

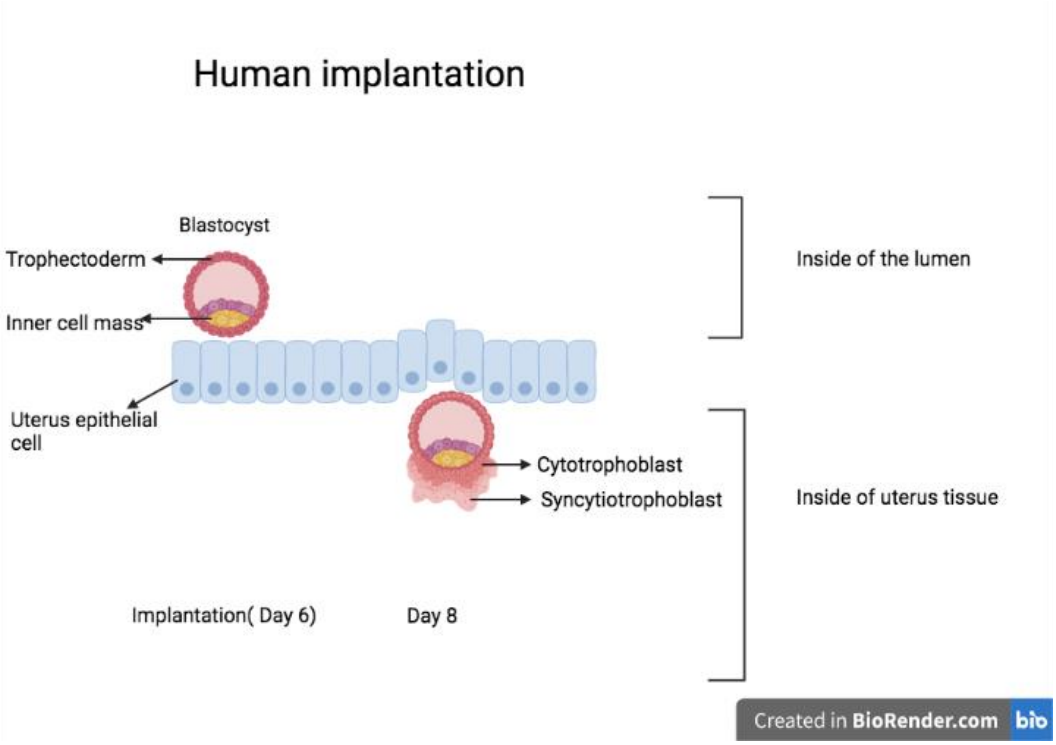


**Figure 1. The implantation of human embryos.** (Created with BioRender.com)

Around day 15, the cytotrophoblast cell gives rise to the second differentiated trophoblast cell type, extravillous cytotrophoblast. These cells are highly invasive, undergoing an epithelial-to-mesenchymal transition to invade deeply into uterine tissues. Invasive extravillous trophoblast home to parental uterine blood vessels and enter them, allowing blood to flow over the developing villi (Figure 2). Fetuses have placental villi (Figure 2) as the membrane which connects with maternal blood and exchanges nutrition and waste. Placental villi have a tree shape structure which maximizes the contact area and increases the ability to exchange with maternal blood. When the placental villi (Figure 2) are matured, the extravillous trophoblast stays in the tips of the villi which are close

to the decidua (Figure 2). In Figure 2, there are lots of immune cells inside of the decidua which protect the embryo from being attacked by maternal immune cells and contribute to the trophoblast invasion and uterine vascular remodeling (Liu, 2017).

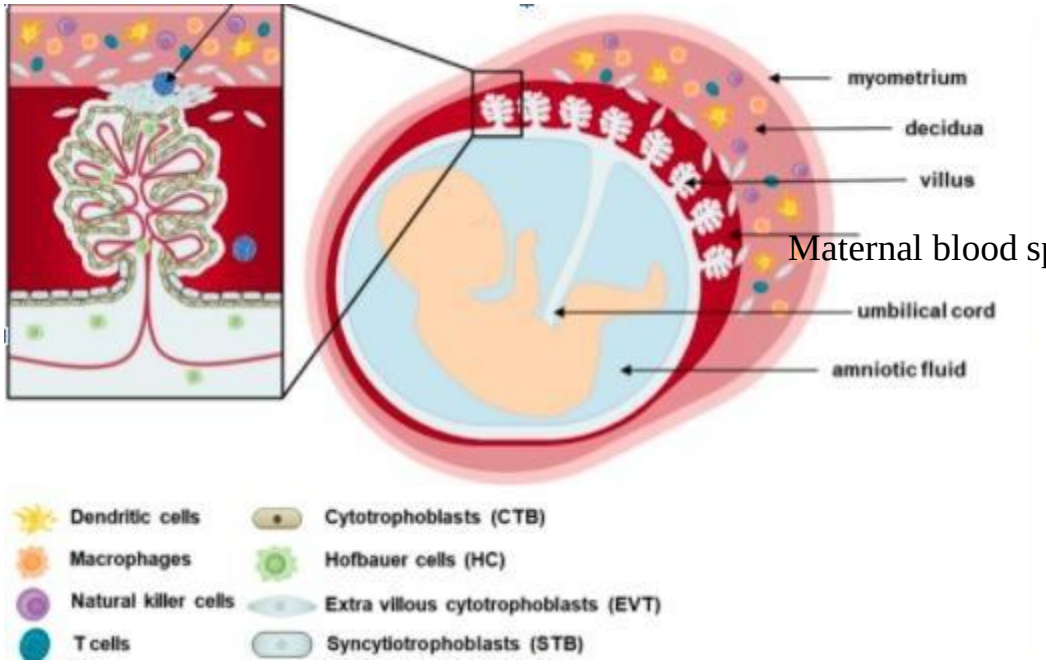


**Figure 2. The placenta in the first trimester.** (Lee, 2020)

## How Uterine Blood Vessels Change

Deep uterine blood vessels are narrow, corkscrew-shaped tubes. They are called “spiral arteries” although physiologically they are arterioles as we will show in the next section. Before day 15, the human uterine blood vessels provide nutrients and oxygen to the fetus (Burton, 2001). Since the need for nutrition and

waste disposal grows, the narrow blood vessel cannot achieve this goal. Healthy blood vessels go through a series of remodeling steps that expand the vessel diameter by disrupting the organized smooth muscle cell, vascular endothelium, and some extracellular matrix (Choudury, 2017). This expansion increases the blood flow to the placenta, lowers the blood pressure, and provides more nutrition for the fetus. At one time, it was thought that the trophoblast itself was responsible for reorganizing and dilating the uterine vessels. The Endovascular cytotrophoblasts invade the decidua, with the same function of the expansion, and promote the blood vessels in the myometrial to remodel (Soares, 2018). But it appears (refs) that early changes in vessel morphology precede trophoblast arrival. In this study, we describe the early remodeling of spiral arterioles in the rat prior to trophoblast arrival.

## Components of Arterioles and Changes in Vessel Remodeling

In this study, we tracked the transformation of arterioles by tracking the migration of several cell types inside the spiral arterioles. Arterioles are small-diameter blood vessels that deliver blood from arteries to capillaries. They selectively constrict and dilate to distribute blood flow into individual tissues and provide 80% resistance to blood flow in the body. Based on all these characteristics, arterioles contribute to the control of the blood pressure and velocity of the maternal blood that flows toward the fetus.

The composition of the wall of the arteriole contributes to our designed method. It usually has three layers: intima, media, and adventitia (Figure 3). The intimal layer has an endothelial cell and basement membrane. The blood flow and pressure can affect the morphology of endothelium. The basement membrane can provide anchoring support to the endothelium. If the endothelium is deformed, the basement membrane exposes to the blood and the vascular smooth muscle can provide signals for anchoring and migration of cells across the vascular wall. The media layer consists of vascular smooth muscle cells which compose most of the media (Figure 3). This layer of smooth muscle controls the vascular diameter through the contraction and relaxation of each cell. In the maternal blood vessel remodeling process, endothelial cells produce and release vasoactive factors and exert on neighboring organized contractual vascular smooth muscles. Then the vascular smooth muscles dedifferentiate and lose their ability to contract (Martinez-Lemus, 2012). The adventitial layer has some fibroblasts surrounded by collagen fibers which provide structural support for the vessel, produce and send out signals to modulate the activity of smooth muscle cells, and contribute to vascular repair (Martinez-Lemus, 2012).

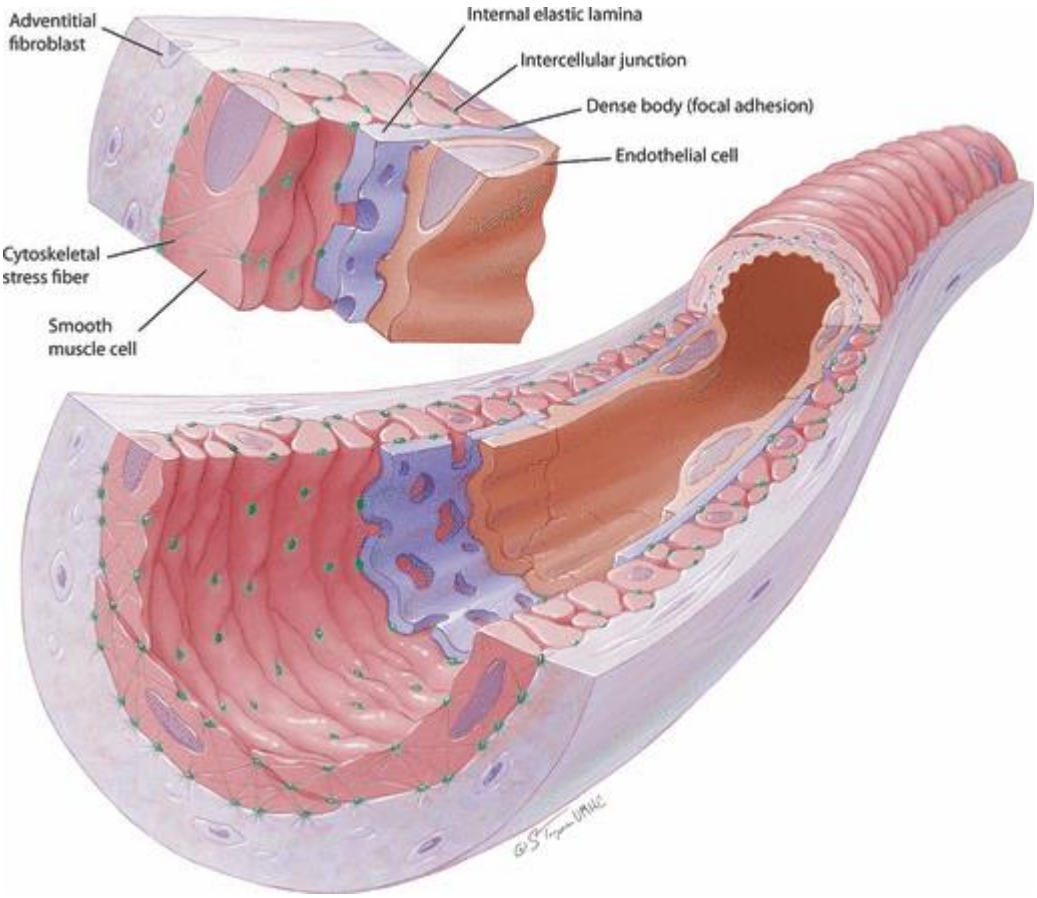


**Figure 3. Schematic representation of an arteriole** (Martinez-Lemus, 2012)

In conclusion, important deconstruction happens before the trophoblast arrives at the vessel; the idea is that parental factors like immune cells may be involved in these early stages. To lay the groundwork for understanding how this process works, in this project we describe the progression of changes in early arterial remodeling.

# Choice of A Model System

We worked on a model system, the Norway rat, Rattus norvegicus. To study the human placenta, researchers can only harvest the human placental tissue from elective abortions. Since those tissues are valuable and hard to get, It is even harder to get tissues with precise development times or the same development time to increase the sample size. Humans often do not know the day or even the week, that conception occurred. Most of the human placenta is harvested after birth which does not match our needs since we focus on the first-trimester development event. Because of the limited sources of getting human placenta tissue in the early period, and the hardness of timing the gestational period, we use a rat model. Anatomically, rats and humans have many features in common. The most crucial one is that they both have a hemochorial placenta in which intrauterine trophoblast cells are invasive directly into the maternal blood (Soares, 2018) (Furukawa, 2019). However, there are some significant differences between these animals. Evolutionally, humans, and rats do not have a close relationship. Humans typically produce one offspring while rats have several offspring. The morphology of the uterus is different as well. Rodents have a two-horned uterus with two long uterine chambers in which the multiple fetuses line up like sausage links. Humans have a single uterine chamber. Rat gestation is 23 days long, while human gestation is approximately 40 weeks long. Even if they have all these differences, the morphology of the placenta in rats and humans are surprisingly

similar in the depth of trophoblast invasion of the uterus, and in the extent to which uterine arterioles are remodeled in early pregnancy. With all these similarities, we choose rats as our animal model.

## Morphology of Rat Placenta and Its Attachment Site

Although rats have a similar deep placenta bed to humans, there are still some morphological differences between these two species. Figure 4 is a cross-section of the rat uterus showing the implantation site with the embryo on gestational day 12. Starting from the top, it is the metrial gland, which is the deep placental bed inside of the mother. It is where maternal blood vessels remodel, which is important for our study. The metrial gland is not a gland at all, but an anatomical zone formed by a split between the longitudinal and circular muscle forming the uterine wall. The second layer is the decidua which connects with the basal zone and is the interface of the maternal and fetal parts of the placenta. It isolates the fetus from the uterus, accumulates glycogen and lipid, and produces hormones. There are several maternal immune cells in decidua which build the maternal immune tolerance at the maternal-fetal interface (Furukawa, 2019). The fetal part has a basal zone, labyrinth zone, and embryo which are not our main focus for this study.

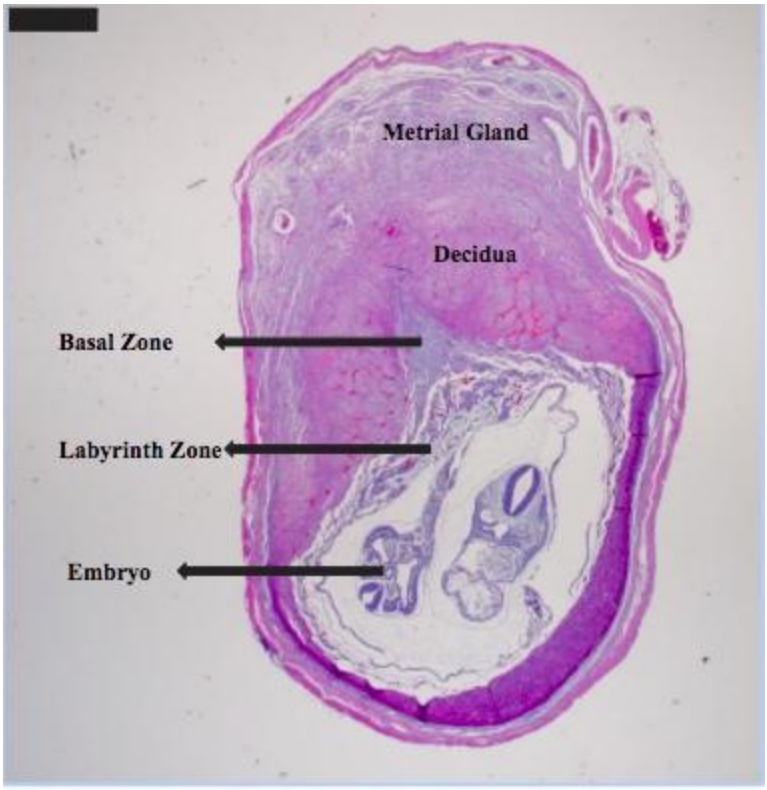


**Figure 4. Cross-section through implantation site showing the embryo, placenta, and deep placental bed in the rat on day 12 gestation.** Stained with Hematoxylin & Eosin. Scale bar:1mm.

## Goals of The Study

This process has been studied, and a lot is known about it such as the component of vessels and how different cells migrate (Choudhury, 2018). However, there are still lots of unknowns such as the relationship between smooth

muscle, endothelial cell, and trophoblast and the extent to which there is really a stage that is trophoblast-independent.

Some simple assign stages based on gestational age (Charalambous, 2011). Others (Lash, 2016) (Smith, 2009) (Choudury, 2018) use the vessel thickness to classify the transformation of the vessel. For a long time, people classify the stages based on the presence and absence of the trophoblast. Still, others follow changes in several cell types (muscle, endothelium, trophoblast) (Smith, 2009) (Choudury, 2018). We combined the classification of these two groups and settled upon our own rat-specific classification stage. We tracked the vessel's smooth muscle thickness and migration of smooth muscle, endothelium, and trophoblast. Through tracking the migration of three kinds of cells, it shows the relationship between them and the certain stages that the trophoblast arrives at. It can contribute to our understanding of the vessel remodeling before and after the trophoblast invasion and figure out the unknowns about this vessel remodeling period. This work is also an initial step to understanding the cell/cell interactions--either between cell types in the arteriole or between arteriole components and cells in the uterine tissue--that contribute to successful remodeling.

# METHODS

## Tissue Collection and Process

Female and male rats (approximately 14 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA). To track the ovulatory cycle, females were given a vaginal saline lavage daily. On the day prior to ovulation, a female was paired with a male overnight for mating. The presence of sperm in a vaginal lavage the following morning confirmed mating. That sperm-positive day was termed "day 1" of pregnancy. Pregnant rats (n = 2/day) were sacrificed on gestation days 9, 10, 11, 12, 13 and 14. Segments of uterine horn were dissected, washed in PBS, and then processed for either hematoxylin and eosin staining, or for immunohistochemistry.

Tissues for H&E staining were fixed in 10% neutral buffered formalin for 24 hours at room temperature, then transferred to 70% ethanol until further processing. Tissues dissected from females on days 13 and 14 of pregnancy were too big for optimal fixation, so in these cases the embryo was removed prior to fixation, leaving just the placental tissues attached to the uterus. We sent tissues in 70% ethanol to the Pioneer Valley Life Sciences Institute for paraffin infiltration and embedding, then we cut the paraffin blocks on a rotary microtome (Leica Microsystems, IL). First, we cut 8um sections through the entire thickness of the embryo's implantation site, generating dozens of paraffin ribbons we laid

out in order. Then retrospectively, using a microscope, we identified the section in the series that lay closest to the exact center of the placenta. We then mounted the sections flanking either side of the center onto glass slides, numbering the slides just before the center from -10 to -1, and the slides just after it from +1 to +10. For each embryo, we thus generated a series of approximately 21 slides on which were mounted near-serial sections passing through the exact center of the placenta. The slides containing these near-serial sections were sent to the Pioneer Valley Life Sciences Institute for (a) hematoxylin and eosin staining and (b) Masson's Trichrome staining (Poly Scientific R&D, Bay Shore, NY).

The H&E stains the nucleus (purple) and the cytoplasm (pink), which helps us to identify the location of the vessel and highlight the smooth muscle layer (pink) around the vessel. The Masson's Trichrome stains the extracellular matrix (blue), especially collagen and fibrinoid. It supports the idea that there are other things around the vessel that might maintain the structure of the vessel during the remodeling.

Tissues for immunohistochemistry were embedded in Optimal Cutting Medium (OCT, Fisher HealthCare, TX) in a bath of isopentenyl cooled over liquid nitrogen. The resulting OCT blocks were stored at -20°C until sectioning. Using a similar schema to what was outlined above, we cut 8um sections on a cryostat (Thermo Fisher, Waltham, MA) and saved sections on a set of up to ten

slides prior to and just after the centermost section. Because frozen sections cannot be saved as “ribbons,” they were mounted on slides. Retrospectively, the center section(s) were identified, and the slides were numbered accordingly.

## Immunohistochemistry

Immunohistochemistry (IHC) combines anatomical, immunological and biochemical techniques to image discrete components in tissues by using primary antibodies to bind specifically to their target antigens in situ. Three primary antibodies used the immunoperoxidase-based staining method. After 15 minutes of acetone fixed and 15 minutes of drying in the humidity chamber at room temperature, any endogenous peroxidase activities were quenched by incubating the sections in 0.3% H2O2 for 12 minutes. Then, nonspecific binding of the primary was blocked by incubating the sections in 10% goat serum for 30 minutes. Primary antibodies were diluted in Tris-buffered saline (TBS) and incubated on the sections either at 4$^{o}$C overnight or 1 hour at room temperature. As a negative control, some sections were incubated in TBS rather than the primary antibody. Goat anti-mouse IgG biotin-SP conjugated (1:500 in TBS, Jackson Immunoresearch) was used as the secondary antibody for 30 minutes. Then we applied peroxidase-conjugated streptavidin (1.5ug/ml, Jackson Immunoresearch) for 30 minutes and Substrate AEC working solution (Vector Labs) for 10 minutes. Washes were 3*5 minutes between each step and performed in TTBS (TBS with 0.1% Tween-20). Sections were counterstained with

hematoxylin (Gill's formulation #1, Sigma Aldrich) for 3 minutes, dehydrated, and mounted with the aqueous mounting medium Glycergel (Dako).

**Table 1. Information of three primary antibodies used in immunohistochemistry.**

| Primary antibody (clone) | Specificity | Manufacturer | Fixation | Dilutions in TBS |
|---|---|---|---|---|
| Mouse Anti-Cytokeratin (C-11) | Trophoblast & another epithelium | Sigma-Aldrich | Acetone | 1:2000/1:10000 |
| Mouse Anti-Actin (ASM-1) | Smooth muscle (N-terminus) | Millipore | Acetone | 1:1000/1:2000 |
| Mouse Anti-PECAM-1 (E-4) | Endothelial cells | Santa Cruz Biotechnology | Acetone | 1:500/1:1000 |

## Image Analysis

Each spiral arteriole within the metrial gland was imaged on an Olympus BX51 microscope (Tokyo, Japan) using Olympus CellSens software. The measurements were done by one assessor (YZ). The maximum and minimum diameter (Minimum diameter is usually perpendicular to the maximum) of the lumen was measured (Figure 5). The mean diameter is the average of these two diameters.

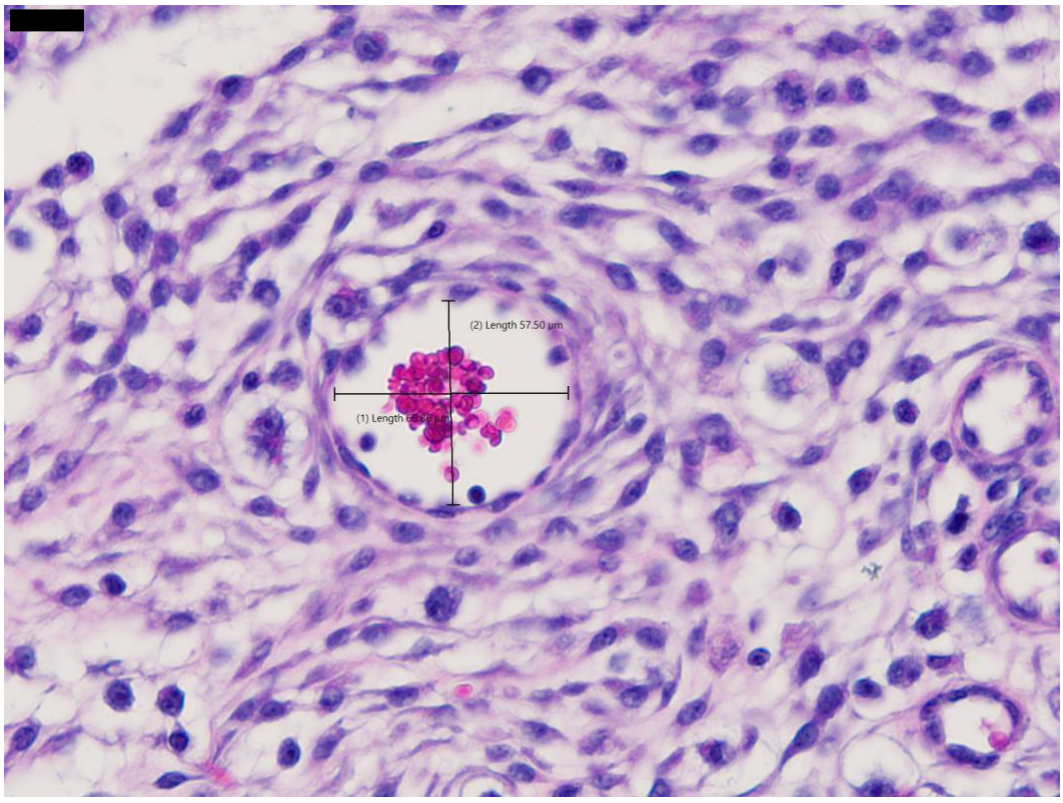


**Figure 5. Measurement of lumen diameter of a spiral arteriole in metrial gland of the rat on day 12 gestation.** Scale bar: 20 ums.

As spiral arterioles remodeling progresses, the remodeling vessel looks a lot like veins. Therefore, a technique was used to define and exclude small veins and arterioles caught in longitudinal sections together with the measurement of the lumen diameter, by limiting the analysis to vessels whose maximum lumen diameter was less than three times their minimum diameter.

With the use of a grid graticule with 5um x 5um (Figure 6) and 10um x 10um (Figure 7) squares in Fiji (ImageJ 2, Version:2.3.0), the thickness of the smooth muscle layer was determined from the hematoxylin & eosin stained sections by placing a graticule over the stained artery at 500x magnification. Smooth muscle media thickness was classified by whether it was 10um and above, between 5 and 10um, or less than 5um.

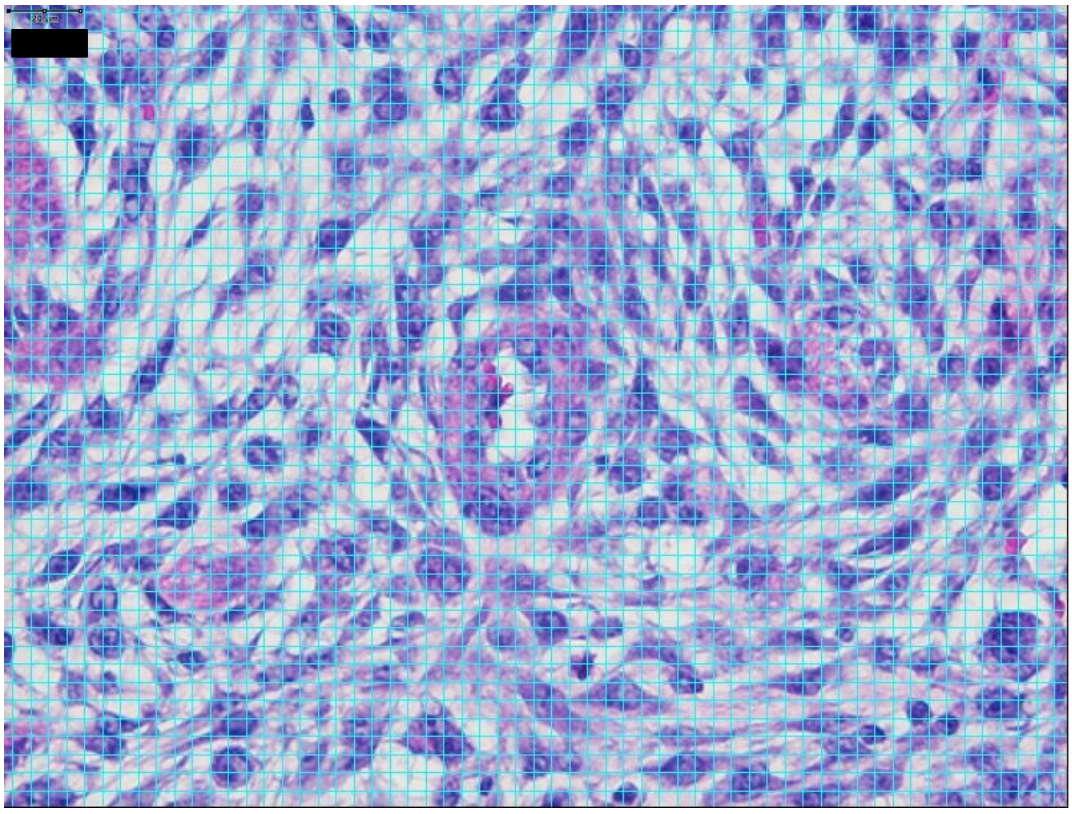

**Figure 6. The measurement of vessel's smooth muscle layer thickness with 5um x 5um squares in Fiji (ImageJ 2, Version:2.3.0).** Metrial gland gestation day 9. Scale bar: 20 ums.

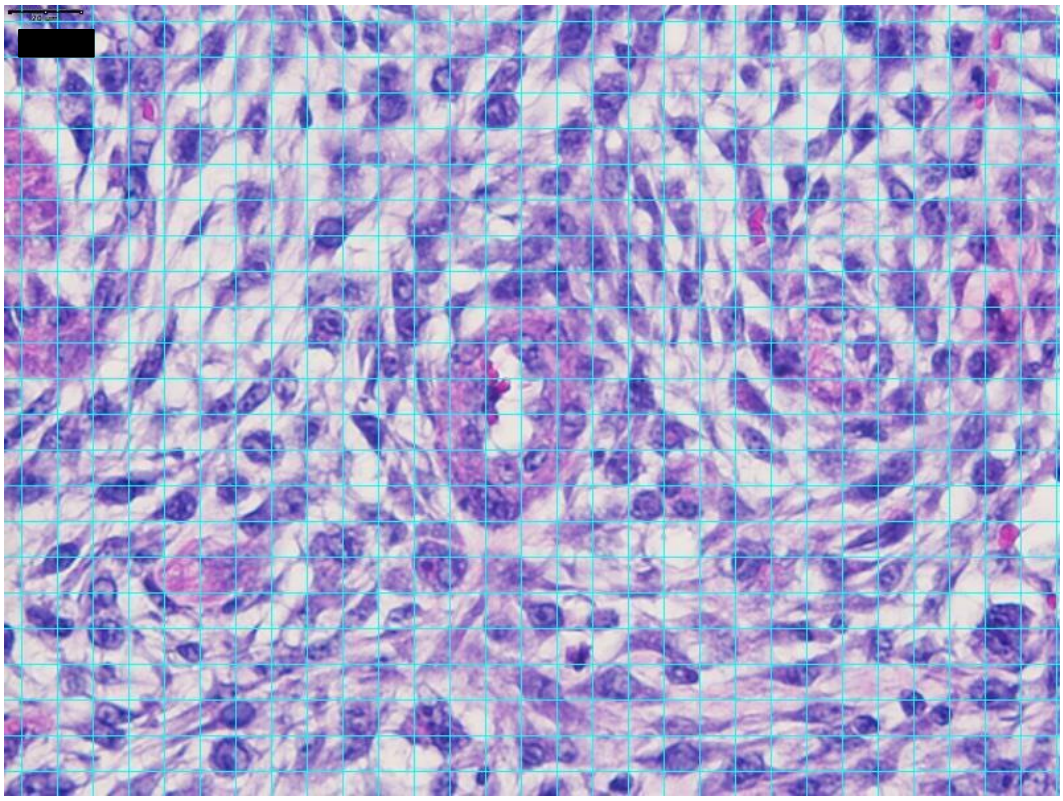

**Figure 7. The measurement of vessel's smooth muscle thickness with 10um x 10um squares in Fiji (ImageJ 2, Version:2.3.0).** Metrial gland gestation day 9. Scale bar: 20 ums.

Near-adjacent tissue sections immunostained with the primary antibodies shown in Table 1 were assessed by taking an image of the corresponding spiral arterioles. The diameter was measured as above. Immunohistochemistry provided a much better resolution of cell type, so we could identify the tissue types in the vessel wall and whether or not they were intact. In the immunohistochemistry stained sections, if the smooth muscle media layer was intact, its thickness was measured, using the method described above for the H&E stained tissues. If the

media layers had broken up and were present in little segments around the vessel, it was not possible to measure its thickness.

While for H&E, we could capture a single vessel, in IHC we had to find the same vessel represented in three different sections, each with clear, high-quality staining for the primary antibody (ASM-1 for smooth muscle, anti-cytokeratin for trophoblast, and anti-PECAM-1 for endothelium). Thus, there were a lot of ways for things to go wrong in analyzing even a single vessel in the IHC data and the number of IHC stain vessels is smaller than the H&E's.

# RESULTS

Using two different staining techniques to visualize thin sections of the rat's deep placental bed on days 9-14 of pregnancy, we analyzed the sequence of anatomical changes in uterine blood vessels. The two staining techniques each had their distinct advantages. H&E stained sections were embedded in paraffin and have really high-quality tissue morphology. Thus, it is possible for us to distinguish different cell types under H&E. The immunohistochemistry sections are frozen into OCT and usually disrupt tissue morphology. However, it highlights various cell types (smooth muscle, endothelium, trophoblast). Our initial intent was to combine these methods together by embedding all the sections in paraffin and stain with H&E and primary antibodies. But not all the cell types can be highlighted in paraffine. Thus, we change to immunohistochemistry.

When we use the gestational days as the measurement at first, some of the spiral arterioles change asynchronously. They do not go through the remodeling process synchronously which can increase the bias of our data if we use gestational days. Through my observation, the part of the metrial gland that is close to decidua and is in the center of the metrial gland goes through remodeling earlier than other vessels around. If we use gestational days, it is impossible to show the difference between vessels near the center and the edges. Thus, I use stages as my measurement.

## H&E and Masson's Trichrome

Table 2 shows that the lumen diameter of the vessel diameter expanded during remodeling, from unremodeled stage 1 (39.2±13.9 um in diameter) to the latest stage we encountered, stage 4 (142.3±78.8 um in diameter). Smooth muscle and endothelium became disorganized and seemed to delaminate starting in stage 2 and 3, changes apparent before the embryo's invasive trophoblast cells had reached the vessels in stage 4. Fibrinoid also appeared (Massion's Trichrome stains) in trophoblast-independent remodeling stages; the trophoblast is only seen around the vessel when there are no endothelium and smooth muscle breaks into pieces. The sample size was smaller on later days of gestation because the remodeling starts at the center of the metrial gland, so the vessel remodeling is asynchronous and when vessels expand their quantity decreases.

**Table 2. Using H&E stained tissues to analyze changes to spiral arteriole in the deep placental bed.**

| | Lumen diameter (um) | Smooth muscle thickness (um) | Endothelium | Trophoblast | Fibrinoid |
|---|---|---|---|---|---|
| Stage 1 (n=79) | 39.2±13.9 | >10 | Multilayered | Absent | Extracellular matrix (absent on vessel) |
| Stage 2 (n=95) | 44.1±17.6 | 5-10 | Swelling and protruding into lumen | Absent | Monolayer |
| Stage 3 (n=51) | 75.3±44.7 | 1-5 | Monolayer | Absent | Monolayer |
| Stage 4 (n=10) | 142.3±78.8 | 0 | Absent | Partly around the lumen | Absent |

In the second column of Figure 8, the pink ring on the H&E sections is the smooth muscle layer, the purple dots inside / on the inner layer of the smooth muscle are endothelial cells, the fluorescent pink dots inside of the vessel lumen are blood cells, the trophoblast is the giant purple cell around the vessel. The images on the H&E column show the morphology changes of the smooth muscle, endothelial cells, and trophoblast. The smooth muscle layer becomes thinner and thinner. The endothelial cells first swell and protrude into the lumen and then disappear. The trophoblast shows up in stage 4 and is partly around the lumen. In the third column, Masson's Trichrome images, the blue lines are fibrinoid. Fibrinoid are present around the vessel throughout the first three stages and disappear in stage 4.

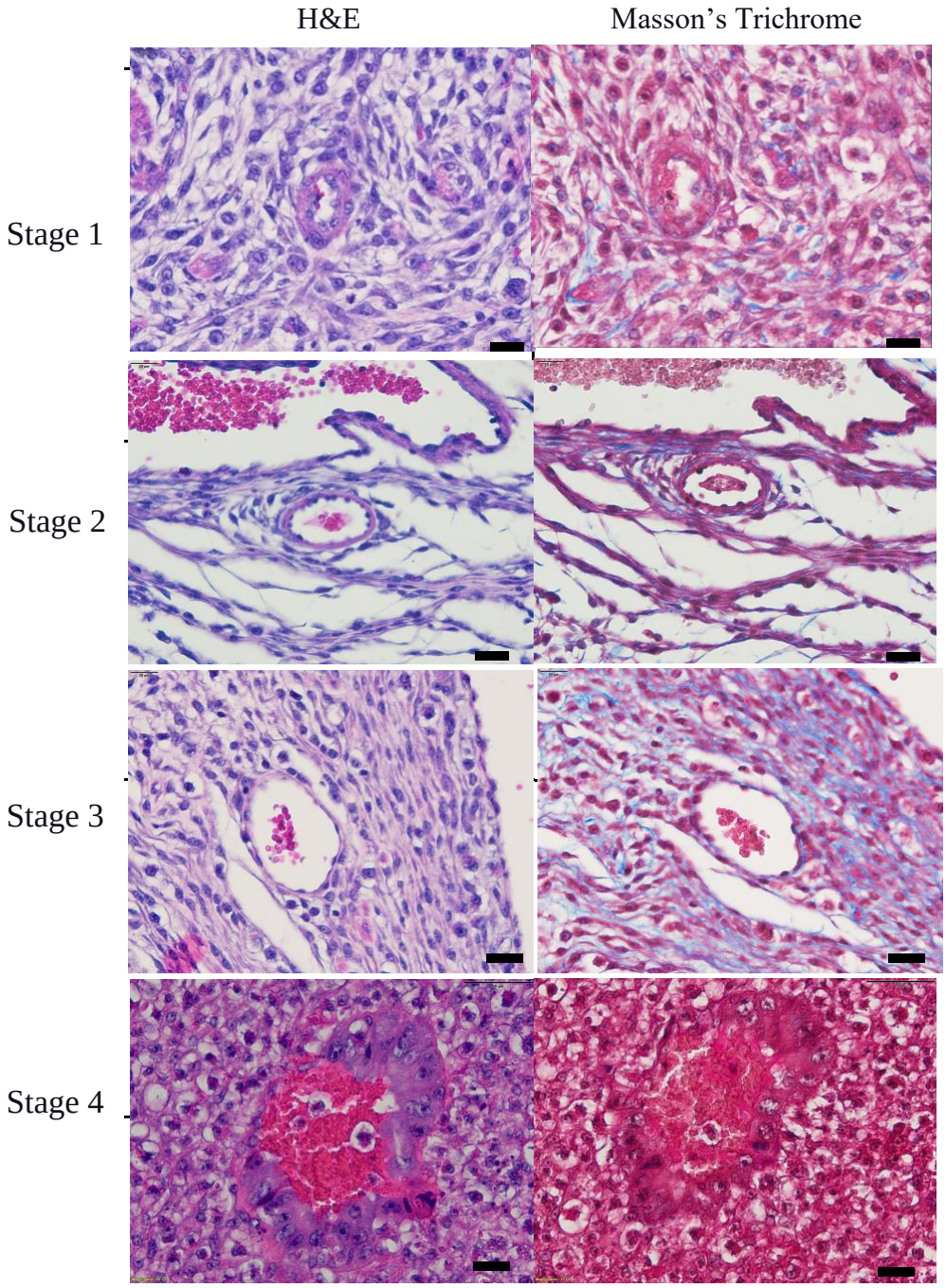


**Figure 8. Paired near adjacent sections through deep placental bed stained with Hematoxylin and Eosin and Masson's Trichrome, showing vessels representing remodeling stages 1 to 4. Scale bar: 20 ums.**

Figures 9 and 10 compare the lumen diameter changes of different classifications (stages and gestational days) which shows that anatomical stages were a more robust way to classify vessels than gestational age. In figure 9, there is a big increase between D12 and D13 and the standard deviation of D12 is extremely big, which shows that the vessel expansion is not correlated with the gestational day. In figure 10, the increases between different stages are smoother which shows that stage is more relative with vessel expansion. The huge standard deviation of figure 9 and figure 10 claim the huge variability of our data.

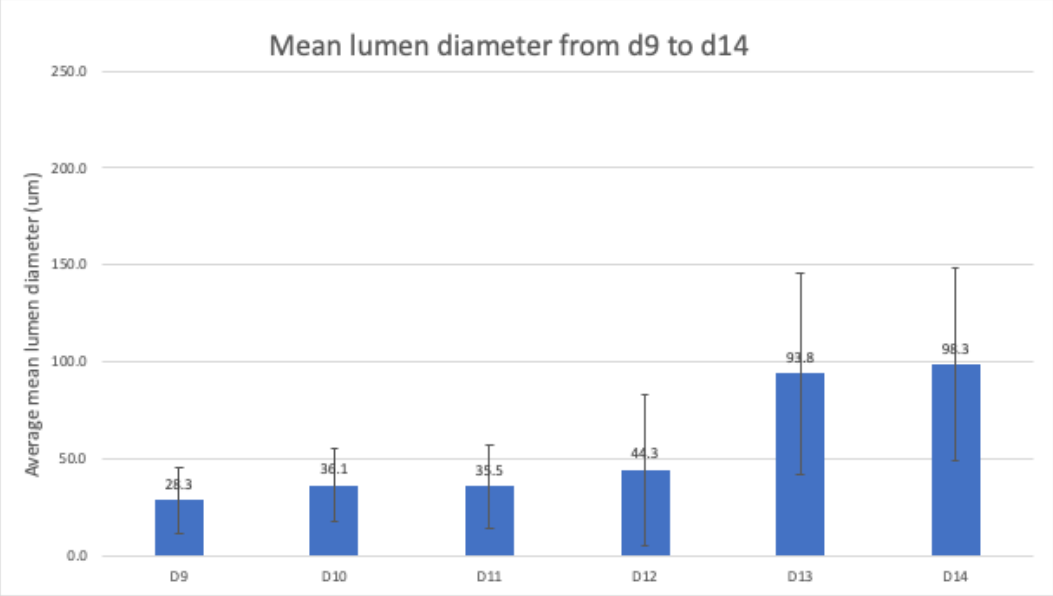


**Figure 9. Average lumen diameter of H&E stained vessels classified in gestational days.** The error bars are the standard deviation.

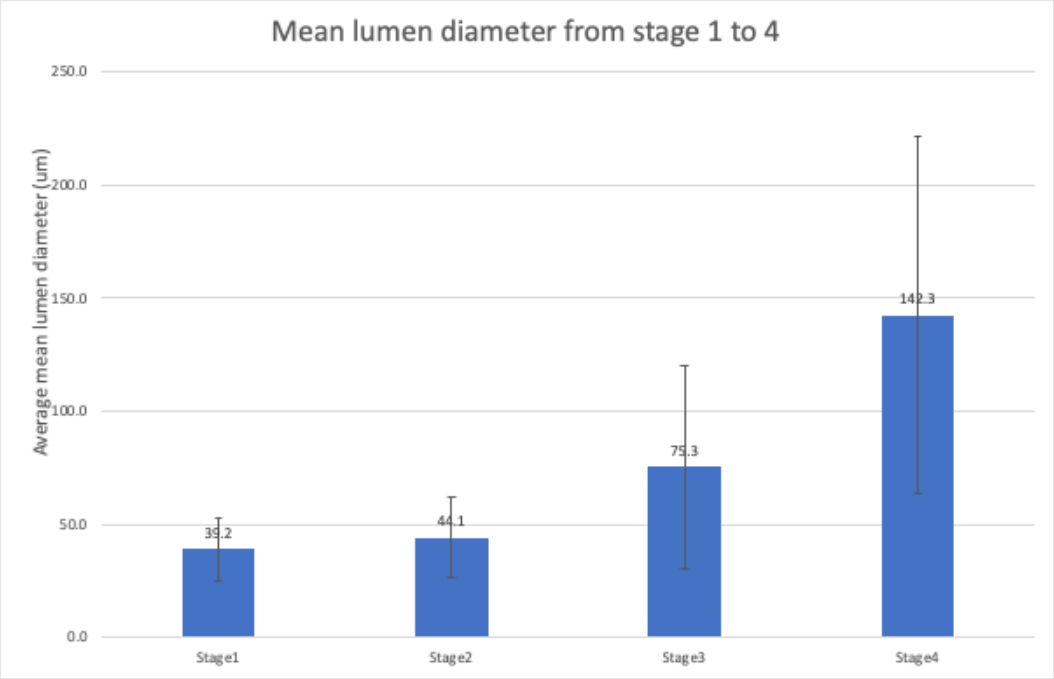


**Figure 10. The average lumen diameter of the vessel, stained with H&E, is classified into stages.** The error bars are standard deviation.

## Immunohistochemistry

The immunohistochemistry stain has a more accurate target than the H&E stain. According to table 3, the vessel lumen diameter increases from 38.3±15.9 um in unremodeled vessels to 342.8±74.4 um in vessels framed by invasive trophoblast cells. Since the smooth muscle breaks into pieces, table 3 only has two different smooth muscle thicknesses. This is different from table 2 where the smooth muscle becomes thinner and does not break. The smooth muscle and endothelium both start to decline after stage 1. Endothelium disappears around stage 3 which is earlier than the smooth muscle (stage 5). Trophoblast starts to

show up at stage 4 which is the same as table 2. From stage 4 to stage 5, the trophoblast progressively increases. In stage 5, the trophoblast fully dominates the vessel lumen.

**Table 3. The variables and classification of the spiral arteriole remodeling with immunohistochemistry sections.**

| | Lumen diameter (um) | Smooth muscle thickness (um) | Smooth muscle | Endothelium | Trophoblast |
|---|---|---|---|---|---|
| Stage 1 (n=31) | 38.3±15.9 | >10 | No breaks | No breaks | Absent |
| Stage 2 (n=43) | 69.2±37 | 5-10 | Small breaks | Small breaks | Absent |
| Stage 3 (n=19) | 150.2±90.3 | • | Partly broken up | Very broken up/ Absent | Absent |
| Stage 4 (n=1) | 328.9 | • | Very broken up | Absent | Partly around the lumen |
| Stage 5 (n=1) | 395.4 | • | Absent | Absent | Fully around the lumen |

Figure 11 to 15 are near adjacent vessels stained with primary antibodies for smooth muscle, endothelium, and trophoblast. A positive stain shows up in red color and the counterstain for the nucleus shows up in blue. Each row represents one vessel under three different immunohistochemistry stains and in most of the figures, there are two or three vessels.

Figure 11 shows the unremodeled situation of three cells (stage 1). All the smooth muscle layers form red rings around the vessel lumen. The endothelium is organized around the inner layer of the smooth muscle. There is no trophoblast at all.

Figure 12 shows the early remodeling of the uterus arterioles (stage 2). The vessel expands compared with the unremodeled vessel. The smooth muscle layer becomes thinner, lighter, or starts to break. The endothelium starts to become lighter, disappear, or stay in its original condition. The trophoblast is mostly not around. However, in the first row of Figure 12, this stage 2 vessel has a trophoblast cell stain in the vessel wall. There are huge variabilities in the disorganized period and all of these anatomies contribute to the breakdown of the smooth muscle and endothelium.

In figure 13 (stage 3), the smooth muscle layer further breaks down, some of them only have several pieces and others have a huge gap inside of the vessel

wall. The endothelium becomes lighter or disappears. Endothelium disappears earlier than the smooth muscle. The trophoblasts are present in the extracellular matrix and absent in the vessel wall. In stage 4 (figure 14), smooth muscle mostly breaks down and only small pieces are left. The endothelial cells are absent, and the trophoblast is partly in the vessel wall. The trophoblast replaces the smooth muscle layer in the vessel wall. In stage 5 (figure 15), there are not any smooth muscle and endothelial cells left. The trophoblast is fully around the vessel.

In conclusion, the smooth muscle layer breaks down and becomes lighter throughout the stages. Similar things happen to the endothelium and it starts to disappear at some of the stage 3 vessels. The trophoblast started to show up around stage 4 and progressively replaced the smooth muscle layer of the vessel.

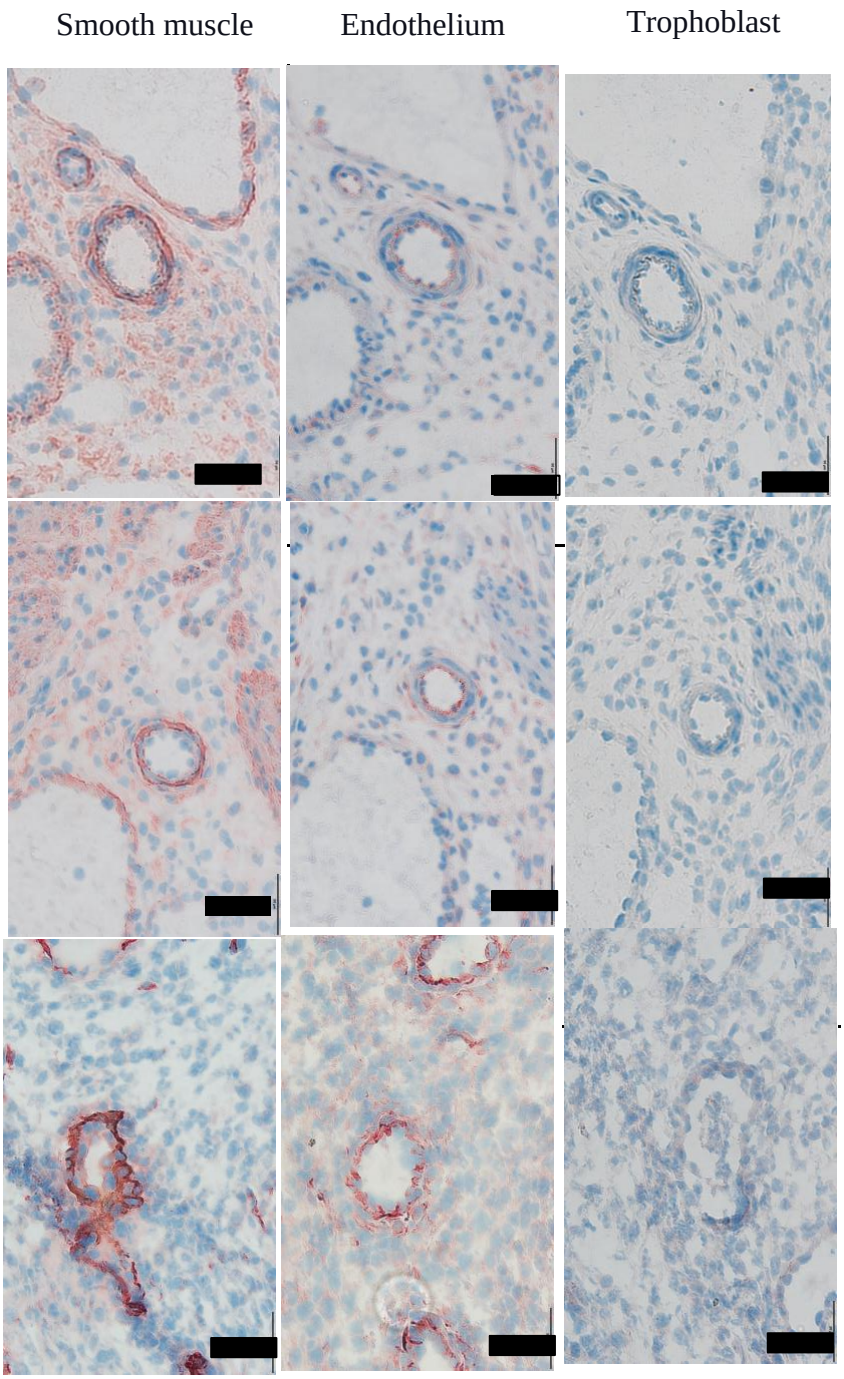


**Figure 11. Near adjacent sections through tissue stained with immunohistochemistry, showing vessels representing remodeling stages 1. Scale bar: 20 ums.**

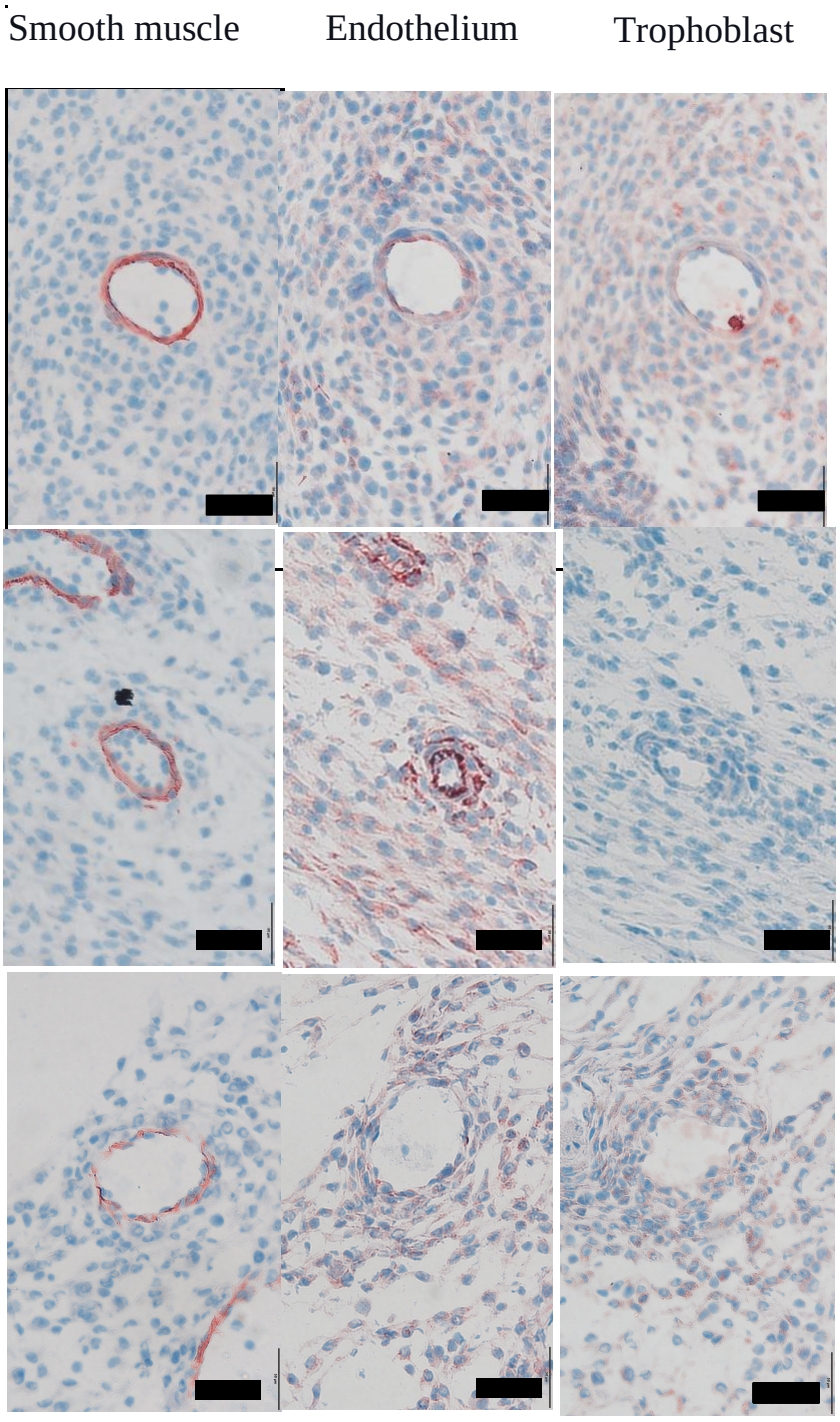


**Figure 12. Near adjacent sections through tissue stained with immunohistochemistry, showing vessels representing remodeling stages 2. Scale bar: 20 ums.**

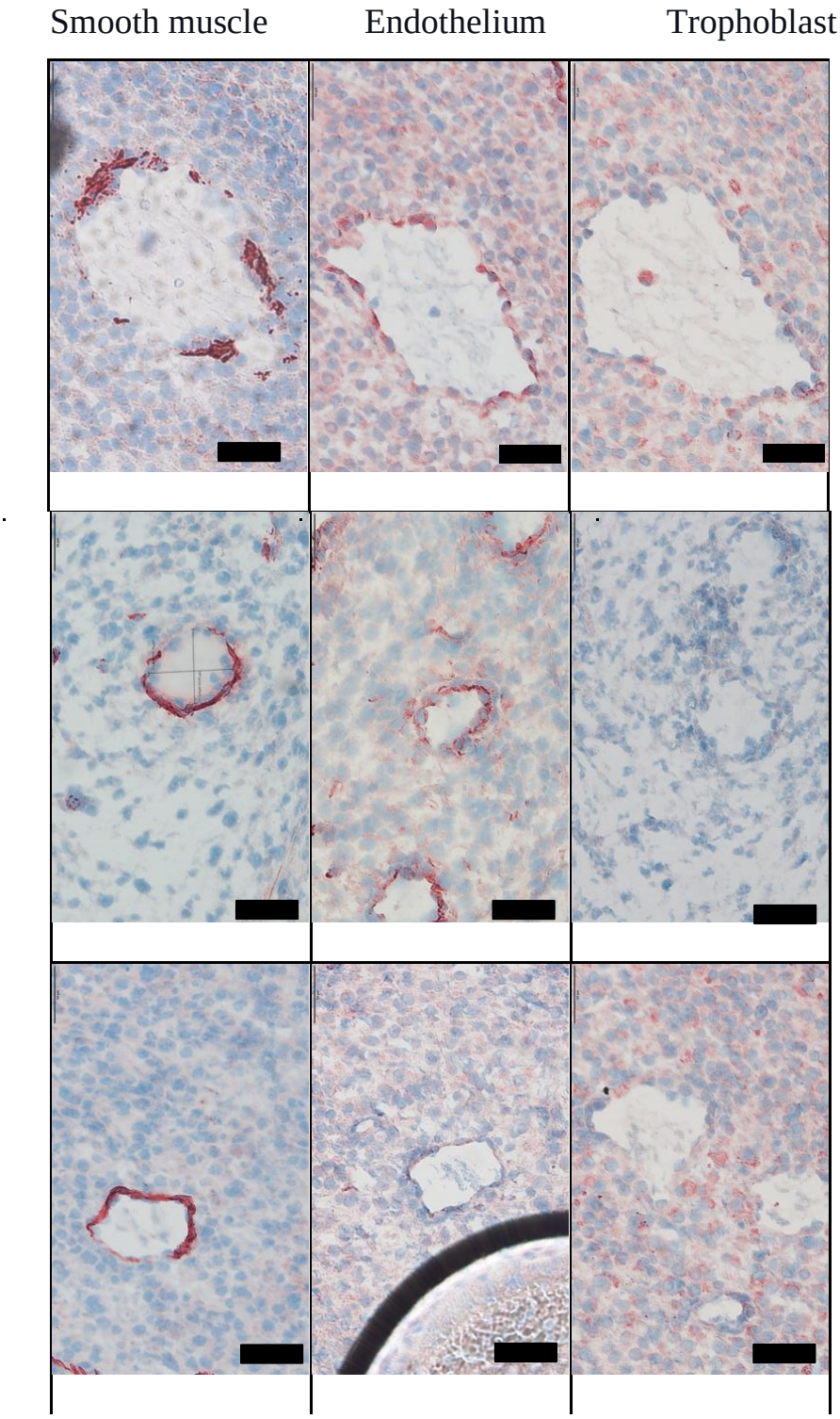


**Figure 13. Near adjacent sections through tissue stained with immunohistochemistry, showing vessels representing remodeling stages 3.** Scale bar: 20 ums.

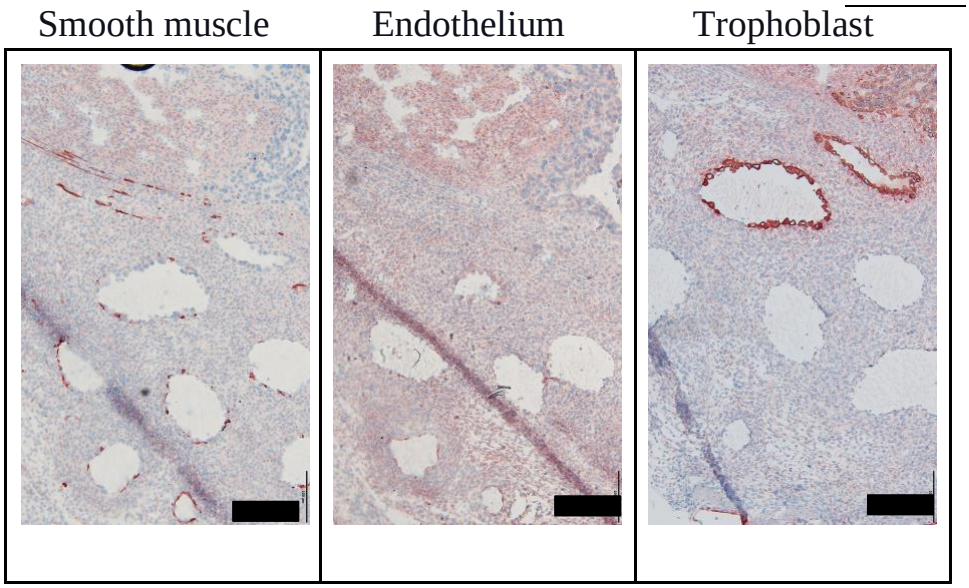


**Figure 14. Near adjacent sections through tissue stained with immunohistochemistry, showing vessels representing remodeling stages 4.** Scale bar: 200 ums.

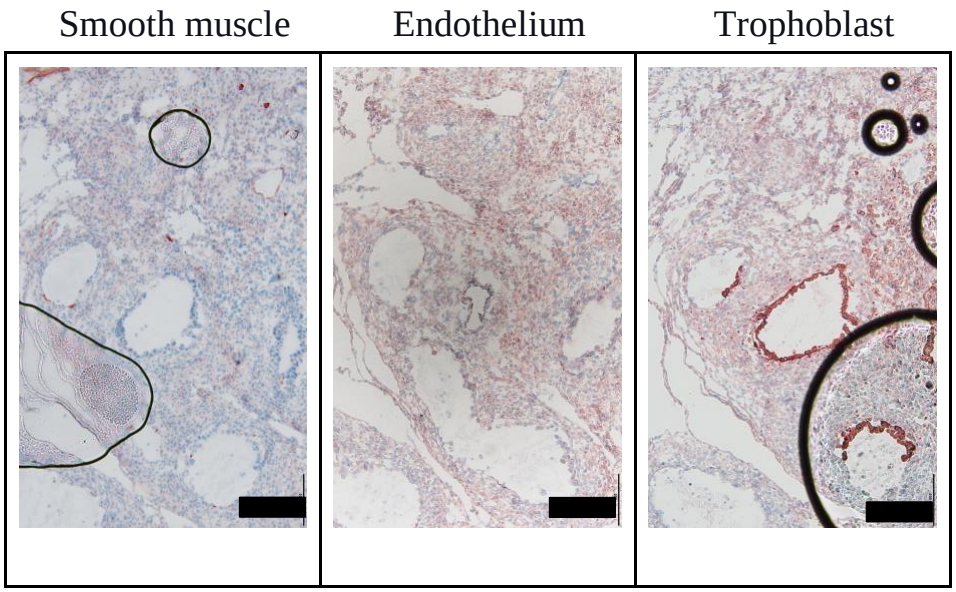


**Figure 15. Near adjacent sections through tissue stained with immunohistochemistry, showing vessels representing remodeling stages 5.** Scale bar: 200 ums.

In the classification process, the smooth muscle is the main characteristic. However, it still has two different measurements (smooth muscle thickness and smooth muscle percentage). Figure 16 compared the lumen diameter of vessels classified by these two measurements and smooth muscle thickness with all three characteristics (smooth muscle, endothelium, and trophoblast). From stage 1 to stage 5, all three bars have a similar mean diameter and increase in the same trend, which demonstrates different measurements do not affect the result.

Figure 17 focuses on the number of vessels in each measurement. The sample size of the smooth muscle thickness and three characteristics are extremely small compared with the other two because it is hard to find the same vessel in all three stains. If we combine figures 16 and 17, the small sample size does not affect the increased tension of the mean lumen diameter throughout the stages at all. Thus, figures 16 and 17 provide extra information about choosing the measurement and sample size.

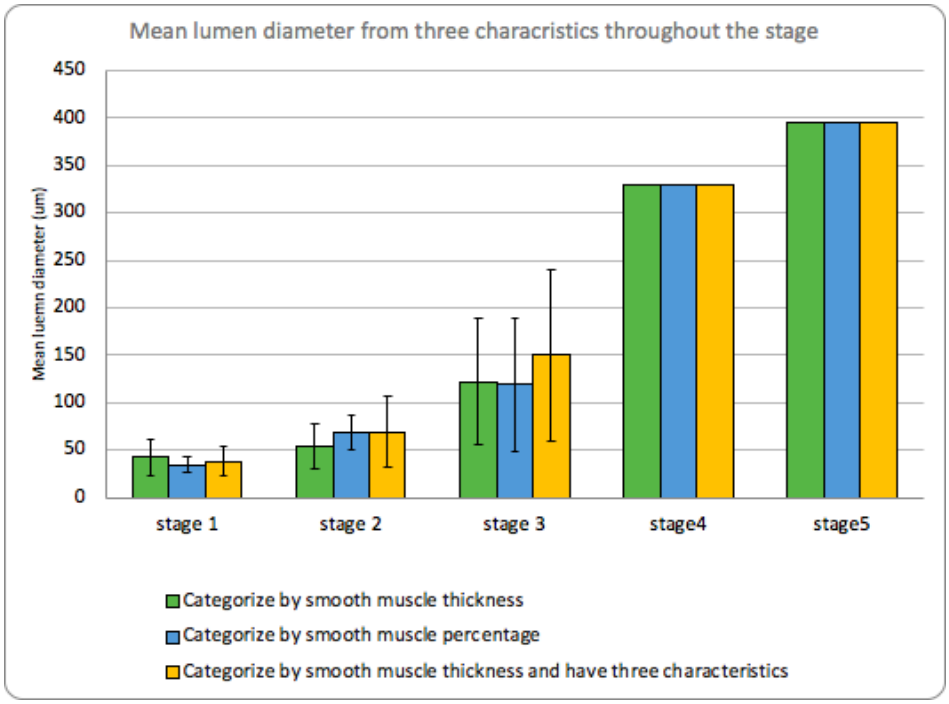


**Figure 16. Mean lumen diameter of three measurements throughout stages.**

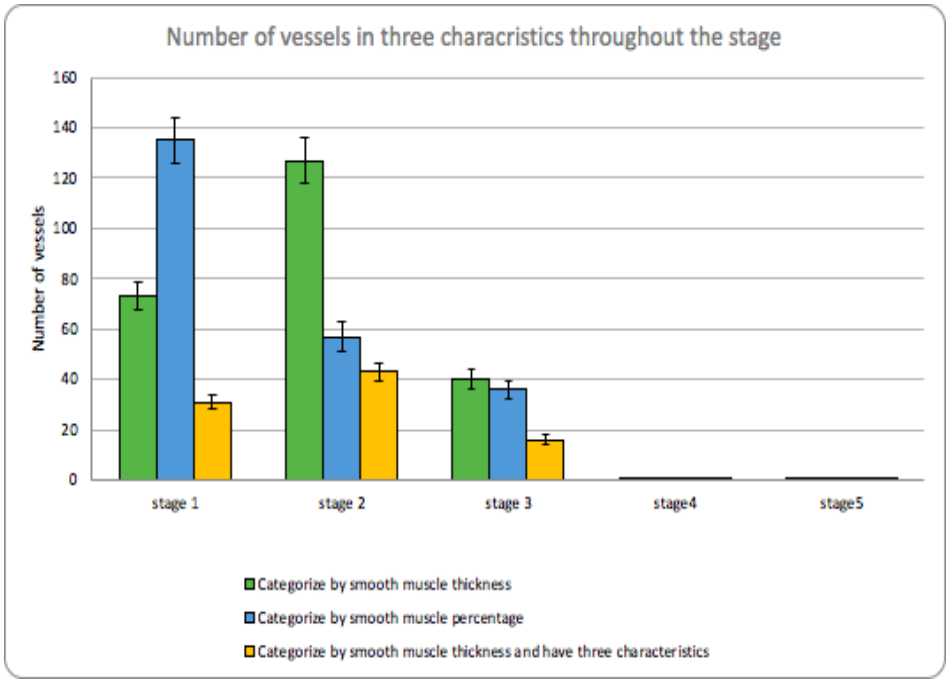


**Figure 17. The number of vessels of three measurements throughout stages.**

Now we can compare and contrast both H&E and immunohistochemistry staining. Figure 18 compares the mean diameter of arteriole diameter under different staining techniques. Because of the different embedded and stain methods, the mean diameter of the immunohistochemistry is higher than the H&E throughout the stages. The H&E staining provides general information on the vessel remodeling and shows some morphology changes in the smooth muscle, endothelium, and trophoblast. However, it cannot show in detail the most important step physiologically. So, immunohistochemistry staining of each target cell type provides more information about the breakup of the smooth muscle layer and helps identify other changes happening in concert with the changes to smooth muscle: when does the endothelium break up, when does trophoblast begin to form part of the wall (how long is the “trophoblast independent” phase). Immunohistochemistry is an important source of the measuring data and is used to make the table of remodeling stages (Table 3).

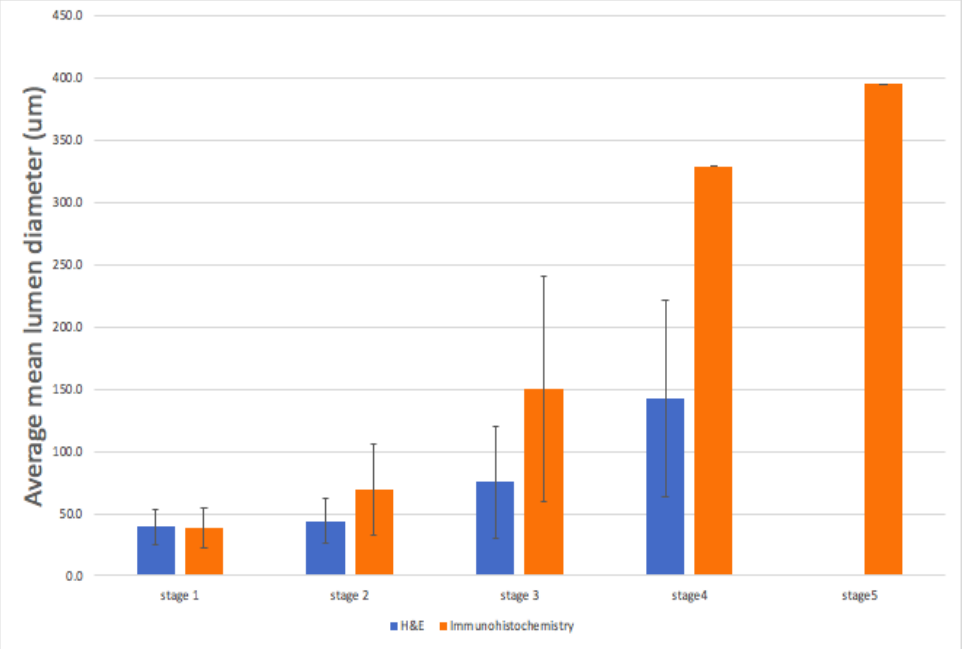


**Figure 18. Mean lumen diameter for H&E stained vessels assigned to their stage (blue) or immunohistochemistry-stained vessels assigned to their stage (orange).**

# DISCUSSION

Through analyzing arterioles in the deep placental bed on multiple days of gestation, we were able to figure out the sequence of anatomical changes that precede the arrival of invasive embryonic cells. The uterine vessels undergo pretty dramatic alterations, maybe in preparation for the arrival of the invasive extraembryonic trophoblast cells. Our categories help us understand the relationship between smooth muscle, endothelium, and trophoblast during the trophoblast independent remodeling.

In rodents, vessel remodeling has been classified by gestational day (Charalambous, 2011). We found this method to be imprecise, as the remodeling is not synchronous within a single metrial gland. They observed a small increase in the spiral artery lumen and a small increase in spiral artery wall thickness. These two results have lots of differences from ours since we observe a huge increase in the lumen diameter and a decrease in spiral artery wall thickness. The difference in the lumen diameter is highly relative to the unremodeled vessels throughout the gestational days and different timelines. They are using gestational day 6.5 to day 10.5. Our experiment is on gestational days 9 to 14. Since they include all the unremodeled small arteries and have an earlier gestational period which makes the overall lumen diameter has a small increase. The difference in the spiral artery wall thickness might relate to the different staining methods. We used the immunohistochemistry method which stains for smooth muscle directly.

They used Masson's Trichrome stain which is hard to distinguish between smooth muscle layers around the vessel and other extracellular matrices.

In humans, where remodeling would occur very early in the first trimester, samples taken following pregnancy termination have been used to assign vessels to stages (Smith, 2009) (Lash, 2016). One of the labs defined their stages based on the disruption of smooth muscle cells and the endothelium (Smith, 2009). They are also tracking the changes of invasive trophoblast and fibrinoid at the same time. The other group utilized smooth muscle thickness as a classification (Lash, 2016). The result might be the difference between different animal models during the remodeling phase or different kinds of stains. To test out which method is better for analyzing the morphological changes of the spiral arterioles in rats, our team followed the stages classification and the gestational days' classification. We analyze the mean vessel lumen diameter, by using two different classifications: gestational days and stages. The gestational day classification does not capture the vessel remodeling process and has lots of variabilities. Especially on day 12, the huge standard deviation shows that the gestational days cannot classify the variability of the vessel during the remodeling periods. The stages classification has a smoother increase which means it is a better classification. Both of these classifications have a huge standard deviation which shows that the vessel lumen diameter does not rely on one of the classifications only. It relates to both of them at the same time which needs a better mathematical model to support this idea.

We also use different embedded materials to figure out the difference made by the stained method. In our histology result, the smooth muscle layer becomes thinner and then breaks. Since H&E is not targeted the smooth muscle cell only, it is hard to use it as evidence. So, we use immunohistochemistry to figure out the migration of certain cell types. The smooth muscle layer starts to break down so quickly (in stage two) and the thickness measurement cannot be used. Although we have a small sample size, it still has a similar mean diameter throughout the stages compared with other measurements.

To further understand the similarity of the remodeling between humans and rats, it is useful to learn about the relationship between smooth muscle, endothelium, and trophoblast in humans and compared it with our rat data. Smith's data shows that the smooth muscle cells and endothelium decline throughout the stages and the trophoblast appears around the later stages (Smith, 2009). In Smith's study, they set up their stages based on the standard thickness and the morphology changes of smooth muscle are their main measurement. Our data also prioritizes the changes of the smooth muscle and shows a similar result to theirs that the smooth muscle cell and endothelial cell are disorganized throughout the stages and trophoblast cells appear in the later stages. The endothelium breaks down quicker than the smooth muscle, which suggests that the breakdown of the endothelium may influence the disorganization of the

smooth muscle. The early vessel remodeling is maternal controlled and trophoblast-independent. The trophoblast contributes to the later period of smooth muscle disruption. These ideas match up with the result that Lash, and Smith have which support the idea that the vessel remodeling process of the human and rat are similar.

We also have data after the trophoblast invasion into the vessel wall to show the relationship between smooth muscle, endothelium, and trophoblast. In our result, there is an overlap between smooth muscle and trophoblast which claims the trophoblast contributes to the vessel remodeling through interaction with smooth muscle in the later stages. Although we mainly focus on the trophoblast independent period of the remodeling, these data work as a counter group which shows the dramatic difference between the trophoblast independent and dependent phase and helps us categorize some events that start before the trophoblast comes.

Researchers were studying the contribution of Nature Killer cells and macrophages to vessel remodeling in the trophoblast-independent phase in humans for a long time (Smith, 2009) (Lash, 2016). Our future studies will investigate the maternal immune cells (Nature Killer and macrophage) and figure out the relationship between trophoblast, immune cells, and smooth muscle in the rat model. Since the sample size is small and vessels have huge variability, it will

be better to increase the sample size which can contribute to our classification. Using other methods to support our result can increase the reliability of this experiment.

# CONCLUSION

In conclusion, our experiment supports the idea that stages are a good classification for vessel remodeling in the rat deep placental bed. Knowing the order in which the vessel deconstructs, we now can investigate the parental-specific factors in the metrial gland that may be responsible.

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