Vrije Universiteit Brussel

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# MOLECULAR MARKERS OF A RECEPTIVE ENDOMETRIUM

Graduation thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in de Geneeskunde

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Academic year 2013-2014

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# ACKNOWLEDGMENTS

I would like to thank Prof. Dr. Christophe Blockeel for providing me the opportunity to make my Bachelor graduation thesis under his supervision and for the interesting topic. I also like to thank him for this valuable feedback on my work. I also would like to thank my advisor Dr. Shari Mackens. I very much appreciate all her advice and support given, as well as the feedback and suggestions made for improving my text.

# 1 INTRODUCTION

Fifteen per cent of couples trying to conceive fail to induce a pregnancy within one year of regular unprotected intercourse (Schulze et al., 1997). Even with the help of artificial insemination, in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), they sometimes fail to get pregnant or suffer from early pregnancy loss. Thirty per cent of embryos are lost prior to implantation, thirty per cent are pre-clinical pregnancy losses (before 6 weeks pregnancy) and ten per cent result in a miscarriage (Salker et al., 2010). Several factors are involved in getting pregnant, many of which still unknown. It is obvious that the embryo and the endometrium are the two key players and a synchronic development between a competent embryo and a receptive endometrium is indispensable for successful implantation. Implantation is the rate-limiting step of artificial reproduction treatment (ART) today. The window of implantation (WOI) opens 6 days after the postovulatory progesterone surge and lasts only about 2 – 4 days (Teklenburg et al., 2010). Implantation itself is a stepwise process. First, there is apposition and adherence of the blastocyst to the endometrium. Next, the luminal epithelium has to be breached. Finally, the blastocyst invades the maternal tissue (Quenby & Brosens, 2013). In this process, embryo and endometrium are of equal importance and a proper crosstalk is essential. So far, a lot of research has been performed on embryonic development, but little is known about the endometrium. Therefore, our focus will be on the maternal side. First, the existing knowledge on the role of the endometrium in implantation will be discussed. Afterwards, a hypothesis will be formulated and hypothetically investigated.

# 2 LITERATURE

First, the micro-array technique will be discussed and a couple of studies performed with this technique will be highlighted. Thereafter, we will take a look at the possibilities with the use of endometrial fluid aspiration instead of endometrial biopsies. The decidualization experiments use another kind of technique to investigate the role of the endometrium and will be discussed next. At the end, the endometrial stem cells deserve scrutiny, and the available evidence about thin endometrium will be discussed.

## 2.1 ENDOMETRIAL TISSUE MICRO-ARRAYS

The endometrium has been extensively studied using the micro-array technique (Edgell, Rombauts, & Salamonsen, 2013). This technique has made it possible to analyze the expression of thousands of genes at the same time in a sample. With this technique, researchers sought for a marker determining the WOI. Failure of the endometrium to gain receptivity is one possible cause of ART failure. A receptivity marker would make it feasible to diagnose this cause. Also the point of time in a woman's cycle to transfer an embryo could be determined. Given the complexity of the implantation process, not a single biomarker but a multiple marker panel was to be expected.

Talbi *et al*. (2006) were one of the first to investigate endometrial samples using micro-arrays. The whole genome molecular phenotype of the endometrium has been analyzed across the cycle. They showed that endometrial samples can be classified by their molecular signature and that they correspond to known phases of the menstrual cycle: the proliferative, early-secretory, mid-secretory, and late-secretory phase. After this study, a lot of research followed

using micro-arrays, especially to find the specific marker to determine the WOI. Several years later, the endometrial gene expression profile in women with unexplained infertility in comparison with fertile controls at the time of embryo implantation was investigated (Altmäe et al., 2010). A clear difference in gene expression between infertile and fertile women was observed. The dysregulated genes of infertile women were involved in cellular localization, transport and transporter activity, most of them located in extracellular regions.

One detection panel that emerged from the research with micro-arrays is the Endometrial Receptivity Array (ERA). Here, 238 genes, differently regulated within the endometrial cycle, are customized on a single array. 134 of these genes represent a specific transcriptomic signature of the receptive phase. The genes of the ERA are coupled to a computational predictor. With this combination it is possible to identify endometrial samples that are within the WOI, regardless of their histological appearance (Díaz-Gimeno et al., 2013; Edgell et al., 2013). Díaz-Gimeno *et al*. (2013) compared the accuracy and reproducibility of the ERA versus standard histological methods and concluded that the ERA scored better on both parameters. To demonstrate the clinical value of the ERA, Ruiz-Alonso *et al*. (2013) formulated the hypothesis that the WOI could be displaced in some patients with repeated implantation failure. They used the ERA as a diagnostic tool to identify this possible displacement of the WOI. The results showed indeed an increased percentage of WOI displacement in patients with repeated implantation failure in comparison with the control group. After diagnosing, they used successfully the concept of personalization of the day of embryo transfer as therapeutic strategy to get these women pregnant.

However, there are many differences between the studies with micro-arrays. This is partly due to the different micro-arrays used for analysis. The differences between the ratios of cells in biopsies are another reason. The endometrium contains many cell types: epithelial and stromal cells, cells of the vasculature and leukocytes. The composition and ratio of these cells shift across the cycle and differs every day. According to the day in the cycle, the biopsy will largely consist of stromal or glandular tissue. Also the composition of the biopsies will be affected by interpersonal differences. Because of these differences, most studies result in finding other markers than those that have been found before (Edgell et al., 2013).

## 2.2 ENDOMETRIAL FLUID ASPIRATION ANALYSIS

Unfortunately, endometrial biopsies cannot be obtained during the WOI in a cycle during which an embryo transfer is planned, since they might disrupt the process of implantation. A solution could be a non-disruptive clinical test. One of the possibilities is aspiration of the endometrial secretions from the uterine cavity during the window of implantation. These glycoprotein-rich secretions are produced by the uterine glands and support the embryo during the pre-implantation period (Boomsma et al., 2008). Van der Gaast *et al*. (2003) has demonstrated that endometrial secretion aspiration does not affect implantation rates. It is a safe method for obtaining sufficient material without damage. Next, endometrial secretions can be analyzed by one-dimensional polyacrylamide gel electrophoresis to demonstrate protein patterns.

Boomsma *et al*. (2008) analyzed aspiration of endometrial secretion, taken prior to embryo transfer, by a multiplex immunoassay to characterize a cytokine profile. They found ten mediators that were detectable in 90 – 100 % of the samples (IL-1β, IL-6, IL-12, IL-18, TNF-

α, VEGF…). They also showed that this technique has no effect on implantation rates. In a later study, Boomsma *et al*. (2009) tried to explore whether a cytokine profile can be identified in endometrial secretions that would make it possible to predict implantation and clinical pregnancy. They analyzed endometrial secretions from 210 women, aspirated immediately prior to embryo transfer, for 17 soluble regulators of implantation. They have found significant associations between low MCP-1 and high IP-10 levels and embryo implantation and between low IP-1β and high TNF-α levels and clinical pregnancy.

Proteomics analyses have also identified a number of possible markers determining the WOI in uterine fluid. Casado-Vela *et al*. (2009) identified 803 proteins in uterine fluid, whereof several proteins are associated with endometrial cyclicity or embryo implantation. This description of the endometrial fluid proteome can be helpful in the future for the study of embryo implantation and for the search to useful markers.

## 2.3 DECIDUALIZATION EXPERIMENTS

Because of the lack on results and agreement between the studies with micro-arrays, some researchers started to use a different method to investigate receptivity markers and to gain insight in the mechanisms of implantation. With the decidualization method, they use endometrial stromal cell cultures. After cultivation, they make the cells decidualize in vitro to perform various experiments. Endometrial stromal cells (ESC) are obtained with a biopsy of the endometrium. After purifying, the cells are brought under cultivation. Next, decidualization of the ESC can be induced by the addition of 8-Br-cAMP and MPA.

The possibilities to investigate receptivity outgoing of these cultures are immense. They can be brought in co-culture with hatched blastocyst, searched for cytokine expression with immunoassay, genes can be knocked-out or knocked-in, etc.

In a study of Teklenburg *et al.* (2010), they investigated the soluble factors involved in implantation. Therefore, they used a human co-culture model, consisting of decidualizing ESCs and single hatched blastocysts. When a normal developing embryo was present, there was no significant effect on the levels of implantation factors secreted by the stromal cells. On the other hand, selective inhibition of these factors was seen with arresting embryos. This could indicate that human ESCs become biosensors of embryo quality upon differentiation into decidual cells. This would give the mother/patient a way of natural selection of embryos to prevent investment in developmentally impaired pregnancies.

Salker *et al*. (2010) formulated the hypothesis that impaired decidualization of endometrial stromal cells predisposes to recurrent pregnancy loss (3 or more consecutive miscarriages). Impaired decidualized ESCs might facilitate delayed implantation of compromised embryos by prolonging the window of endometrial receptivity. To test this hypothesis, the levels of prolactine (PRL), a decidual marker, and prokineticin-1 (PROC-1), a cytokine that promotes implantation, were determined. First, they measured the transcription levels in endometrial biopsies from recurrent pregnancy loss patients and controls. Compared to the controls, recurrent pregnancy loss was associated with significantly higher endometrial PROC-1 mRNA levels and significantly lower PRL levels. Next, they established primary cultures and decidualized them over 8 days. After 4 days of differentiation, the levels of PRL were significant lower in the group with recurrent pregnancy loss then in the control group. The

PROC-1 levels kept growing in both groups, until day 8. From then on the expression in the control group declined markedly, in contrast to those in the group with recurrent pregnancy loss. These observations support the hypothesis that impaired decidualization of endometrial stromal cells predisposes to recurrent pregnancy loss.

Kuroda *et al.* (2013) investigated the importance of glucocorticoids upon decidualization. They show that decidualization of the ESCs is associated with the induction of 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1). 11βHSD1 catalyzes the conversion of cortisone to his active form cortisol. They also found a high expression of glucocorticoid receptors (GR) in undifferentiated ESCs, but upon decidualization the receptor was down-regulated. This was in contrast to the mineralocorticoid receptor (MR) levels, which were up-regulated upon decidualization. To identify MR- and GR-dependent genes in differentiating ESCs, they used siRNA-mediated knockdown with genome-wide expression profiling. GR-repressed genes were transcriptional repressors involved in epigenetic programming. MR knockdown showed changes in lipid droplet formation and retinoid metabolism. All the results together suggest the formation of a corticosteroid gradient upon decidualization at the feto-maternal interface.

## 2.4 STEM CELLS

Tissues with high tissue regeneration, like intestinal epithelium, bone marrow and epidermis, have their own adult stem cells. Also in the endometrium, which has more than 400 cycles of regeneration in a woman's life, adult stem cells were discovered. Both layers of the endometrium, the functional and the basal layer, contain endometrial stem cells. There are small populations of epithelial progenitor cells and mesenchym stem cell (MSC)-like cells. These cells serve the monthly cycles of regeneration, differentiation and shedding of the uterine lining (Gargett, Nguyen, & Ye, 2012).

Currently, robust identifying markers for endometrial epithelial progenitor cells are not yet found, although a couple of candidates are under investigation (Gargett et al., 2012). For endometrial MSC, Schwab *et al*. (2007) identified co-expression of two specific markers, CD146 and PDGF-Rβ. The $CD146^{+}PDGF\text{-}R\beta^{+}$ stromal cells are multipotent and differentiate into typical mesodermal lineages. They express a typical MSC phenotype and are located around blood vessels in both endometrial layers. Later, a single marker was discovered to identify endometrial MSCs, W5C5 (Gargett et al., 2012). The properties of the $W5C5^{+}$ cells are similar to the properties of the $CD146^{+}PDGF\text{-}R\beta^{+}$ population. With this single marker it is possible to use magnetic bead sorting instead of flow cytometry sorting, improving the yield of viable cells.

Menstrual cycling and endometrial regeneration clearly being essential for receptivity, abnormal functioning of endometrial MSCs or/and their niches could be an underlying cause of endometrial malfunction and ART failure.

There is already one study done about abnormal functioning of endometrial MSC cells and ART failure by Murakami *et al*. (2013). They explored a connection between obesity (Body Mass Index, age and reproductive outcome) and endometrial MSCs (abundance and cloning efficiency). Their results show higher first trimester pregnancy losses in obese subjects. Also the number of $W5C5^{+}$ cells and their cloning efficiency were lower compared to subjects with

a normal Body Mass Index. They suggest that the regenerative capacity and plasticity of the endometrium of obese women is suboptimal.

### 2.5 THIN ENDOMETRIUM

The endometrium needs an adequate growth to allow successful implantation. Low implantation rates have been described for women with a thin endometrium (Miwa et al., 2009), although this is still a topic of discussion (Dain et al., 2013). A thin endometrium could be caused by impairment of the normal endometrial growth, but the exact mechanism is not clarified. A number of treatments have been tried to stimulate the endometrial growth in patients with a thin endometrium (estrogens, low dose aspirin…), but without much success (Miwa et al., 2009).

## 3 HYPOTHESIS

At the Centre for Reproductive Medicine of the Universitair Ziekenhuis Brussel, physicians are often confronted with patients whose endometrial thickness remains below limits to perform an embryo transfer with substantial chance of success. Having done a comprehensive literature study on endometrium, endometrial receptivity, eMSCs and human embryo implantation, I formulated following hypothesis:

**Can a problem at the level of the eMSCs be responsible for ART failure in case of thin endometrial lining?**

To test this hypothesis, first we have to know which is the best way to identify eMSCs. The two possible markers are CD146 PDGF-Rβ and W5C5. We will compare both methods in order to retrieve the optimal results. Next, we can investigate the actual hypothesis. Therefore we will compare the relative number of stem cells and their functional capacity between thin and normal endometrial linings.

## 4 MATERIAL AND METHODS

### 4.1 ETHIC STATEMENT AND PATIENT SELECTION

Approval for the research will be requested to the "Commissie Medische Ethiek" of the UZ Brussel. Written informed consent will be obtained from all participating subjects. To investigate the hypothesis, patients will be divided in two groups depending on their endometrial lining thickness. Each group will contain about ten persons aged 23 – 44 years; these numbers are in line with other studies (Miwa et al., 2009; Schwab & Gargett, 2007). We will compare the relative number of stem cells and their functional capacity between thin and normal endometrial linings. Patients waiting for embryo transfer with an endometrial thickness ≥ 8 mm will be in the normal-thickness group and those with an endometrial thickness < 8 mm will be in the thin-thickness group. This threshold thickness is defined based on a prior study (Miwa et al., 2009), in which they used IVF-ET data. This data showed

significant lower pregnancy rates of patients with an endometrial thickness $< 8$ mm in comparison to patients with an endometrial thickness $\geq 8$ mm. We will exclude patients with uterus abnormalities in the thin-thickness group, because otherwise our results could be distorted. The endometrium will be measured with a vaginal ultrasonography and tissue will be collected at the time of planned embryo transfer using a pipelle biopsy (Miwa et al., 2009).

## 4.2 PREPARATION OF HESC SUSPENSION

First, single cell suspentions of human endometrial stromal cells (HESCs) will be generated as described by Murakami *et al* (2013). Biopsies will be washed in a medium of DMEM/F-12. Next, they will be finely minced and enzymatically digested with collagenase (0.5 mg/ml) and deoxyribonuclease type I (0.1 mg/ml) for 1 hour at 37°C. The dissociated cells will be filtered through a sterile 40um cell strainer. Undigested fragments (mostly glandular clumps) will be retained on the cell strainer. The stromal cells and blood cells will pass through. To remove the erythrocytes, the stromal single cell suspensions will be centrifuged with a Ficoll-Paque PLUS. The stromal cells will be collected in the medium/Ficoll-Paque PLUS interface. The medium/Ficoll-Paque PLUS interface will be aspirated and washed with DMEM/F-12 medium.

## 4.3 $CD146^+PDGF^+$ DETECTION

To detect the $CD146^+PDGF^+$ cells, fluorescence activated cell sorting (FACS) will be used as previously described in an article of Schwab *et al* (2007). CD146 antibody and PDGF-Rβ antibody will be used to label the endometrial stromal cell suspensions ($1 \times 10^7$ cells/ml). Thereafter, to make the anti-body labeled cells visible, they will be labeled with fluorescein isothiocynanate (FITC)-conjugated anti-mouse $IgG_{2a}$ (50 µg/ml) and phycoerythrin (PE)-conjugated anti-mouse $IgG_1$ (10 µg/ml). The cells will be incubated with allophycocyanin (APC)-conjugated anti-CD45 (10 µg/ml) to label leukocytes and resuspended in 5% fetal calf serum/phosphate-buffered saline (FCS/PBS) containing propidium iodide (PI, 10 µg/ml) to label dead cells. This will be sorted with FACS on a MoFlo flow cytometer using Cyclops SUMMIT software. The cells will be analyzed for CD146-FITC and PDGF-Rβ-PE expression. The leukocytes and dead cells will be excluded from the analysis.

## 4.4 $W5C5^+$ DETECTION

The $W5C5^+$ cells will be detected using Magnetic Bead Selection, like described by Masuda *et al* (2012). The endometrial stromal cell suspensions (up to $1 \times 10^7$ cells/100µl) will be incubated with phycoerythrin (PE)-conjugated W5C5 antibody (10 µl/$10^7$ cells) on ice for 30 min in 0,5% fetal calf serum in PBS (Bead Medium). Then, the cells will be incubated with anti-PE magnetic-activated cell sorting MicroBeads for 30 min, also on ice. Cell suspensions (up to $1 \times 10^8$ cells/500µl) will be applied onto Miltenyi columns in a magnetic field, followed by washing the column with 500µl Bead Medium three times. Most $W5C5^-$ cells will pass through the column. The magnetically labeled $W5C5^+$ cells will be retained on the column. By removing the columns out of the magnetic field, the $W5C5^+$ cells can be flushed out with 1ml of Bead Medium. After the Magnetic Bead separation the cells can be counted and the percentages can be calculated.

### 4.5 IN VITRO COLONY-FORMING ASSAY

To determine the cloning efficiency, an in vitro colony-forming assay will be performed. The CD146$^+$PDGF$^+$ and CD146$^-$PDGF$^-$ cells and the W5C5$^+$ and W5C5$^-$ cells will be seeded at a clonal desity of 50 cells/cm$^2$ onto fibronectin-coated 60 mm cultured dishes. They will be cultured in a growth medium of DMEM/F12 supplemented with 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS), 1% L-glutamine, 1% antibiotic-antimycotic solution, 2 μg/ml insuline, 1nM estradion and 10 ng/ml basic fibroblast growth factor. The first medium change will be after 7 days. Thereafter, changes will be done every 3-4 days. Microscopic monitoring will be necessary to secure that the colonies will be derived from single cells. After 15 days, the colonies will be terminated and stained with hemotoxylin. Clusters of ≥ 50 cells will be counted. The cloning efficiency (CE) will be calculated with the formula:

$$\text{CE (\%)} = \frac{\text{number of colonies}}{\text{number of seeded cells}}$$

(Masuda et al., 2012; Murakami et al., 2013; Schwab & Gargett, 2007).

### 4.6 STATISTICAL ANALYSIS

Data will be analyzed with GraphPad PRISM software and SPSS. Normal distribution will be tested using histograms and the Kolmogorov-Smirnoff test. Unpaired t test or ANOVA will be performed on the data, depending on the number of groups compared. Data will be presented as mean±SEM. Pearson correlation will be used for calculation of associations between variables. Results will be considered statistically significant when $P<0,05$ (Masuda et al., 2012; Murakami et al., 2013; Schwab & Gargett, 2007).

# 5 DISCUSSION

### 5.1 COMPARISON BETWEEN CD146$^+$PDGF$^+$ DETECTION AND W5C5$^+$ DETECTION

To find the best method to identify eMSCs, we will compare the relative number of eMSCs found in both methods as well as their cloning efficiency. After performing FACS and Magnetic Bead Selection to find respectively the CD146$^+$PDGF$^+$ and W5C5$^+$ cells, we can count the number of possible eMSCs found in each method. To determine if all the CD146$^+$PDGF$^+$ and W5C5$^+$ cells are definitely eMSCs, we will perform an in vitro colony-forming assay to calculate their cloning efficiency. If the cloning efficiency is high, most of the cells will be eMSCs. However, if the cloning efficiency is low, a lot of differentiated cells will be filtered together with the eMSCs. This will then indicate that the used method is inadequate to identify only eMSCs. We will also determine the cloning efficiency of the CD146$^-$PDGF$^-$ and W5C5$^-$ cells. In this case, the cloning efficiency has to be low. If it would be high, it would mean that the technique had not identified all the eMSCs. We expect that the cloning efficiency of CD146$^+$PDGF$^+$ is higher than those of CD146$^-$PDGF$^-$. Idem ditto with the cloning efficiency of W5C5$^+$ and W5C5$^-$. Both methods are not yet perfect so there will

still remain eMSC in the CD146$^{-}$PDGF$^{-}$ and W5C5$^{-}$ groups and visa versa. Our goal is to find the method that filters most eMSCs.

If one method has better results on both parameters, it is clear that this method will be chosen.

If one method filters a higher relative number of eMSCs than the other, but the other method has a higher score on the cloning efficiency, we will have to decide which parameter we consider the most important. The technique that filters the most eMSCs (in relative numbers) of all the cells in the HESC suspension may be a first indication that this is the best method to identify eMSCs, however we have to be careful with this interpretation. It is possible that one of the methods filters a lot of positive cells, but these cells are not necessary eMSCs. The cloning efficiency will give us a better picture on the level of stemcellness. Therefore, we will prefer the method with the highest cloning efficiency.

If there would be no difference between the two methods, we will prefer W5C5$^{+}$ detection to isolate eMSCs. The protocol of Magnetic Bead Selection is simpler than flow sorting. Because of the use of two markers with CD146$^{+}$PDGF$^{+}$ detection, only flow sorting can be used or you would need to perform two sequential magnetic bead selections, which makes the protocol more difficult. Flow sorting and two times magnetic bead selections may also affect the cell viability (Masuda et al., 2012).

## 5.2 STEM CELLS IN THIN ENDOMETRIUM VERSUS NORMAL ENDOMETRIUM

We foresee four possible results comparing the relative number of stem cells and their functional capacity between thin and normal endometrial linings.

1. **The relative number of stem cells is equal for thin and normal endometrium, but the functional capacity of thin endometrium is lower.**

In this case the number of eMSCs is not the problem. The fact that they don't function like they should gives the impairment of the endometrium. The next question to ask would be: "Why is their functioning incomplete?" A possible explanation is already given by Miwa *et al*. (2009). They showed that a thin endometrium was correlated with a high blood flow impendence of the uterine radial artery. Other explanations could be a defect in certain receptors or pathways, insensitivity to signal molecules… Further investigation will be necessary to clarify the whole mechanism.

2. **The relative number of stem cells is lower in thin endometrium than in normal endometrium, but their functional capacity is equal.**

Here, the eMSCs function optimal, but there are too little stem cells to build a proper endometrial wall. Even if the cloning capacity is at his maximum, it will be difficult to create an endometrium thick enough. In the future, stem cell transplantation could be a possible solution. More investigation will be necessary to determine the reason why there are less eMSCs. Are there less eMSCs from birth or did they decrease during life?

3. **The relative number of stem cells and their functional capacity is lower in thin endometrium than in normal endometrium.**

In this case both parameters are affected. There are not enough eMSC to make the endometrium grow as thick as necessary. Also, the stem cells that are present do not function like they should. For these eMSC, it will be twice as hard to create an adequate endometrium. Also in this case, future investigation will be needed.

4. **The relative number of stem cells and their functional capacity is equal between thin and normal endometrium.**

This means that no difference is found between thin and normal endometrium. In this case, this study will not be able to formulate an explanation for the incomplete growth of the endometrium in women with a thin endometrium. Further studies will be necessary to provide answers to this question.

# 6 CONCLUSION

A lot of women are confronted with fertility problems. After a literature study, it became clear that there are still a lot of questions around fertility and the mechanisms of getting pregnant. The endometrium plays an important role in getting pregnant. It needs an adequate growth to allow successful implantation. Low–implantation rates have been reported for women with a thin endometrium, but it remains a topic of discussion. A thin endometrium could be caused by impairment of the normal endometrial growth, but the exact mechanism is not yet clarified. To gain more insight in the mechanisms of thin endometrium, we formulated the following hypothesis: "Can a problem at the level of the eMSCs be responsible for ART failure in case of thin endometrial lining?". Next, we discussed the material and methods that can be used to test the hypothesis. First, we have to know which is the best method to identify eMSCs. For this, there are two possible markers: CD146 PDGF-Rβ and W5C5. We will compare both to see which one is the best. Next, we can start investigating the actual hypothesis. Therefore, we will compare the relative number of stem cells and their functional capacity between thin and normal endometrial linings. After reflecting about the possible outcomes, we can conclude that in all the possible cases more questions will rise. Further investigation will be necessary to provide full answers to these questions, but performing the suggested experiments will definitely be a good starting point.

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