***In vivo* visualization of uterine mast cells by two-photon microscopy**

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Abstract

Transgenic mice expressing fluorescent proteins in specific cell populations are widely used for the study of *in vivo* behavior of these cells. We have recently reported that uterine mast cells (uMCs) are important for implantation and placentation. However, their *in vivo* localization in uterus before and during pregnancy is unknown. Herein, we report the direct observation of uMCs *in vivo* using double-transgenic C57BL/6J *Mcpt5-Cre ROSA26-EYFP* mice with high expression of enhanced yellow fluorescent protein in MC protease 5 (*Cma1* (*Mcpt5*))-expressing cells by intravital two-photon microscopy. We were able to monitor MCs live *in utero* during the murine estrous cycle and at different days of pregnancy. We demonstrated that uMCs accumulated during the receptive phase of the female (estrus) and persisted in large numbers at early pregnancy stages and around mid-gestation and declined in number in non-pregnant animals at diestrus. This intravital microscopy technique, including a custom-made microscope stage and the adaption of the surgical procedure, allowed the access of the uterus and implantations for imaging. The introduced application of intravital microscopy to C57BL/6J-*Mcpt5-Cre ROSA26-EYFP* mice offers a novel and powerful *in vivo* approach to further address the evident relevance of uMCs to reproductive processes with obvious clinical implications.

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Introduction

Besides the well-documented role of mast cells (MCs) in IgE-mediated allergic responses (Galli & Tsai 2012), MCs appear now as key effector cells influencing a variety of physiological as well as pathophysiological processes (Bischoff 2009, Anand *et al.* 2012). Not only do they contribute to innate and adaptive immune responses (Galli *et al.* 2005, St John & Abraham 2013), but also they are directly involved in non-immunological processes including tissue remodeling and angiogenesis (Varayoud *et al.* 2004, Bosquiazio *et al.* 2007, Theoharides *et al.* 2010). Their effects are mainly mediated by granule-stored mediators such as metalloproteases, MC-specific proteases, tissue plasminogen activator (tPA), vascular endothelial growth factor (VEGF), transforming growth factor β (TGF β) (Galli *et al.* 2005) and galectin 1 (Gal1, Woidacki *et al.* 2013) that are released upon activation. We have recently reported that MCs migrate from the periphery to the uterus responding to hormonal fluctuations

(Jensen *et al.* 2010). This has already suggested their importance for pregnancy, a period characterized by great hormonal changes. In a mouse model, *Kit* (*c-Kit*) deficiency, which is associated with a lack of MCs including uterine MCs (uMCs), was related to impaired implantation and placentation that negatively influenced fetal growth (Woidacki *et al.* 2013). We found these effects to be mediated by Gal1 as bone marrow-derived MCs (BMMCs) from WT mice but not from *Lgals1* (*Gal1*)-deficient mice could rescue the impaired reproductive phenotype (Woidacki *et al.* 2013). The *in vivo* behavior of MCs at the fetal–maternal interface is, however, largely unknown. As there were some controversial reports on the role of MCs in pregnancy (Salamonsen *et al.* 1996, Varayoud *et al.* 2004, Menzies *et al.* 2012, Woidacki *et al.* 2013), it is of vital importance to localize uMCs at the fetal–maternal interface and to later dissect their behavior and interaction with other cells live.

Transgenically modified mice with fluorescent-labeled MCs using MC-specific Cre recombinase/loxP-mediated recombination are very useful for the

visualization of MCs *in vitro* but particularly *in vivo* (Luche *et al.* 2007, Scholten *et al.* 2008). In 2001, the production of *Cre* reporter alleles that express enhanced yellow fluorescent protein (EYFP), a color variant of the green fluorescent protein, was described (Srinivas *et al.* 2001). In this mouse model, EYFP was inserted into the ubiquitously expressed *ROSA26* locus, framed by a loxP-flanked stop sequence. Crossing these *ROSA26*-EYFP strains with transgenic strains expressing *Cre* either in a ubiquitous or in a cell-specific pattern would now allow monitoring *Cre* activity in living tissues (Srinivas *et al.* 2001). Scholten *et al.* (2008) were the first to describe the combination of the *Mcpt5*-*Cre* transgenic mouse line crossed with the *ROSA26*-EYFP *Cre* excision reporter strain. Due to the lack of *Mcpt5*-*Cre* transgene expression in MCs of the mucosal type, EYFP signal was detected exclusively in connective tissue-type MCs (Dudeck *et al.* 2011) and the serine protease *Mcpt5* showed unique tissue specificity for connective tissue-like MCs (Lützelshwab *et al.* 1998).

Two types of MCs are known: mucosal and connective tissue-type MCs. This classification is based on phenotypic differences rather than on their tissue localization (Metcalf *et al.* 1997, Schwartz & Huff 1998). Additionally, for rodents, a third population is described. These cells show characteristics of both mucosal and connective tissue-type MCs (Schwartz & Huff 1998, Michaloudi & Papadopoulos 1999, Woidacki *et al.* 2013). Unfortunately, to the best of our knowledge, no model is currently available in which both types of MCs can be visualized *in vivo*. Although the percentage of *Mcpt5*-expressing MCs from isolated uterine cells ranged between 5 and 20% (Woidacki *et al.* 2013), double-transgenic C57BL/6J-*Mcpt5*-*Cre* *ROSA26*-EYFP mice with high expression of EYFP in *Mcpt5*-expressing cells emerge as a powerful tool for the visualization of uMCs live and *in vivo* by two-photon microscopy. Here, the simultaneous absorption of two photons, which precedes the emission of the fluorescence, is harmless to the tissue, avoids photobleaching and enables a better tissue penetration (Paoli *et al.* 2009). Based on these methodological advantages, two-photon microscopy became a preferred technique for intravital imaging (Zipfel *et al.* 2003, Olivieri & Tadokoro 2013). Until recently, the visualization of cells *in vivo* at the fetomaternal interface was hampered by the fact that *in vivo* microscopy was not established for the uterus or the placenta. Recently, we have succeeded in visualizing the fetomaternal interface *in vivo* by modifying the technique for intravital imaging (Zenclussen *et al.* 2012, 2013).

In this study, the application of this intravital technique to C57BL/6J-*Mcpt5*-*Cre* *ROSA26*-EYFP mice provided direct observations of MC presence and localization within the virgin and pregnant mouse uteri live and *in vivo*. This information opens up vast new possibilities to understand the behavior and functional relevance of uMCs in health and disease.

Materials and methods

Animals

Mcpt5-*Cre* mice (Scholten *et al.* 2008) were crossed with R26 StopFLOX-EYFP reporter mice (Srinivas *et al.* 2001). *Cre* reporter strains were produced by targeted insertion of EYFP and ECFP into the *ROSA26* locus, which express EYFP in cells with a deleted loxP-flanked stop element. These mice were then housed at the Central Animal Facility at the Otto-von-Guericke University Magdeburg, Medical Faculty, with a 12 h light:12 h darkness cycle. In all experiments, mice at the age of 10–16 weeks were used to avoid age-dependent influences on their reproductive phenotype (Talbert 1971, Parkening *et al.* 1978, Holinka *et al.* 1979). All procedures were in accordance with the institutional guidelines of Magdeburg, and the study was previously approved by the Landesdirektion Dessau (42502-2-978 Magdeburg). This is in agreement with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, USA.

A vaginal lavage was performed to determine the exact state of the estrous cycle by analyzing the content and type of cells in the vaginal smear. For this, the vagina was gently washed with 20 µl 0.9% sodium chloride solution (Berlin-Chemie, Berlin, Germany). For identification of the state of the estrous cycle, the cells were distributed on an object slide and analyzed by light microscopy with a total magnification of 200× (ECLIPSE-S, Nikon, Düsseldorf, Germany). For *in vivo* imaging of the virgin uterus, we focused on diestrus and estrus as we previously found these two phases to be the time points with major differences in the number of MCs between each other (Woidacki *et al.* 2013). For *in vivo* imaging of the pregnant uterus, C57BL/6J-*Mcpt5*-*Cre* *ROSA26*-EYFP mice were mated allogeneically with BALB/c males. The day of vaginal plug was set as day 0 of pregnancy. Pregnant females were separated from the males upon plug detection. *In vivo* imaging of the uterus was performed on gestational days 0, 2, 5, 8, 10 and 14.

Processing of the uterus/implantations for the two-photon microscopy technique

Animals were anesthetized by s.c. injection of ketamine (CP-Pharma, Burgdorf, Germany) (125 µg/g body weight of mouse) and Rompun (Bayer Health Care, Bayer Vital GmbH) (50 µg/g body weight of mouse). After s.c. anesthetization, mice were ventilated through a tracheal tube with a mechanical small animal respirator (Mini-Vent, Hugo Sachs Elektronik, March-Hugstetten, Germany) receiving a mixture of 0.6 vol% isoflurane (Baxter, Unterschleißheim, Germany) and oxygen for narcosis using a Univentor 400 Anaesthesia Unit (Univentor, Zejtun, Malta).

Animals were kept on top of a heating pad at 37 °C and hairs at the abdomen were removed using depilatory cream (Veet, Mannheim, Germany). Fluorescence labeling of blood vessels was done by retro-orbital injection of 100 µl Rhodamine B (10 mg/ml, Sigma-Aldrich) or 12.5 µl QTracker 655 nontargeted QDots (Invitrogen) dissolved in 80 µl aqua ad injectabilia (Berlin-Chemie). The uterus could be accessed by a lateral section at the abdomen. The female mouse and its uterine horns were stabilized on a microscope stage (custom-made design,

Hasomed, Magdeburg, Germany). One of the uterine horns was carefully exposed and fixed using an aluminum foil fixator (self-made), which was placed under the uterine horn without disrupting it. Uterine contractions were reduced by drenching the organ with caffeine (20 mg/ml, Sigma–Aldrich Chemie GmbH). Caffeine was provided continuously in dissolved form using caffeine-soaked cotton. We have shown previously that the addition of caffeine can prevent uterine muscle contraction without any effect on blood circulation (Zenclussen *et al.* 2013). Low-melting agarose (2% in PBS, Peqlab, Erlangen, Germany) was carefully placed around and above the whole uterus to keep the organ at physiological temperatures. After preparation, a heater with a glass-covered opening was placed on top. The temperature probe connected to this heater was placed in very close proximity to the organ where a temperature of 37 °C was maintained throughout the experiment.

Imaging of uMCs by two-photon microscopy

For imaging of the uterus, two-photon microscopy was performed using a Zeiss LSM-710 microscope (Carl Zeiss, Jena, Germany) with simultaneous detection via three external non-descanned detectors. The microscope was equipped with a MaiTai TiSa laser (Newport/Spectra-Physics, Darmstadt, Germany) on an AxioExaminer upright stage and a water-dipping lens (NA 1.0, magnification $\times 20$). Illumination was performed at a wavelength of 850 or 950 nm. EYFP-positive MCs were detected at 500–550 nm emission. Blood vessels were labeled either with Rhodamine B and detected with a bandpass filter of 565–610 nm or with QTracker 655 non-targeted QDots detected at 640–710 nm. Tissue structure was detected by its second-harmonic generation (SHG) signal below 480 nm based on the non-centrosymmetric structure of collagen (Williams *et al.* 2005). Images were recorded as z-stacks overarching the whole uterine horn with 3 μ m Z-spacing using Zen software 2009 (Carl Zeiss). For 3D reconstruction, Volocity software (Perkin Elmer/Improvision, Waltham, MA, USA) was used. Images are representative for three observations in total.

Quantification of uMCs

The number of MCs was determined in representative z-stacks of uteri from virgin females either at diestrus or estrus of the estrous cycle ($n=5$ /each) or during pregnancy (days 0, 2, 5, 8, 10 and 14) ($n=3$ per stage) using Zeiss LSM Image Browser software (Carl Zeiss Microimaging GmbH). The mean number of MCs was calculated as the total number of MCs per recorded z-layer of one z-stack. Statistical analysis was performed by ANOVA test, followed by *post hoc* Tukey's multiple comparison test.

Results

Two-photon microscopy represents a powerful tool for the visualization of uMCs live and in vivo

We first visualize uMCs intravitaly in surgically exposed virgin and pregnant murine uteri. Application of two-photon laser scanning microscopy allowed the live imaging of uMCs up to depths of ~ 200 μ m in double-

transgenic C57BL/6J-Mcpt5-Cre ROSA26-EYFP mice, depending on biological divergences such as pregnancy stage and technical implementation. Compared with the methods applied to fixed tissue, the intravital microscopy technique allowed us to detect uMCs live.

Caffeine was used for reducing the intensive uterine contractions at a concentration of 20 mg/ml. Its application allowed high-quality recording of images, 3D renderings and videos. However, day 14 of pregnancy represented the maximum limit for imaging because at later pregnancy stages not only the uterine contractions but also fetal movement are present, and it becomes impossible to reduce fetal and uterine movements without a surgical manipulation of the uterus.

For imaging of the highly blood-perfused, sensitive and contracting uterus, tissue immobilization was evaluated by a new surgical technique. This was performed in accordance with the procedures described previously (Zenclussen *et al.* 2013). One horn of the murine uterus bicornis was carefully exposed (Fig. 1A), fixed by a self-made aluminum foil fixator and permanently embedded in caffeine-soaked cotton rolls (Fig. 1B) to reduce uterine contractions. As MCs are mainly located in the uterus rather than in placental tissue (Woidacki K, Zenclussen AC, 2010; unpublished observations), we avoided damage to the uterine wall, a procedure shown to be beneficial for imaging of the placenta (Zenclussen *et al.* 2012). Therefore, the focus was on the mesometrial side of the intact uterus (Fig. 1C) and one implantation (Fig. 1D), with special emphasis on the zone between the uterus and the decidua basalis (Fig. 1D). The permanent moistening of uterine tissue was assured by the addition of low-melting agarose. The implantation to be analyzed was covered by the heating plate containing an opening that allowed the access to the zone of interest (Fig. 1E).

Monitoring of vitality after surgery and during intravital imaging was assured by visualization of the blood flow. In our first experiments, different laser wavelengths were tested to excite simultaneously both EYFP and blood flow. In further preliminary works, Rhodamine B, a red fluorescent blood dye, was used. Implementation of both criteria – simultaneous excitation of EYFP and Rhodamine B at the maximum with one certain wavelength and usage of higher and therefore more tissue-gentle and harmless wavelength – revealed that Rhodamine B was not optimal for our setup (data not shown). Therefore, QTracker 655 nontargeted QDots were administrated by retro-orbital injection which allowed the visualization of both blood flow and MCs during the whole imaging process using deep red filter with an absorption wavelength between 640 and 760 nm for detection of emission of QDots. Visualization of uMCs, blood flow and SHG signal at once was then facilitated by excitation at a wavelength of 950 nm. These fine-modulated settings allowed us to monitor MCs live and *in vivo* within the virgin as well as

Figure 1 Surgical procedure to make the uterus accessible for applying intravital microscopy. (A) The anesthetized mouse was placed dorsally on a heating plate. One uterine horn of the murine uterus bicornis was exposed surgically to make the implantation accessible for imaging. (B) The basic fixation of the uterus and the implantations respectively was ensured by a custom-made microscope stage. After the fine fixation by a self-made fixator, the uterus/implantations were permanently drained by applying cotton rolls that were soaked with caffeine to reduce uterine contractions during the imaging process. (C) The focal area was on the mesometrial site, more precisely (D) on that zone at which the fetus is in tight contact with the maternal uterine tissue. (E) The implantation was covered by a heating plate containing an opening that allowed the access to the zone of interest.

pregnant uteri of C57BL/6J-*Mcpt5*-Cre *ROSA26*-EYFP mice in which *Mcpt5*-expressing MCs are positive for EYFP. This is the first report not only in visualizing the pregnant decidua live at different days but also in reporting the presence of MCs in the uterus of a live mouse, confirming the presence of these cells at the fetal–maternal interface.

MCs are present in the virgin uterus and accumulate during the receptive phase of the estrous cycle, the estrus

By using intravital microscopy together with two-photon microscopy, we could confirm *in vivo* that uMCs are mainly located in close proximity to blood vessels (Figs 2A, B and 3A, B, C, D, E and F). Furthermore, this method allowed the assessment of the quantitative distribution of uMCs along the murine estrous cycle (Fig. 2A and B). By using flow cytometry and histology, we have recently reported that the number of MCs peaked during the receptive phase of the female, namely at estrus when the uterus is prepared for nidation, while the lowest numbers were observed during diestrus (Woidacki *et al.* 2013). By using intravital microscopy for detection of EYFP-positive uMCs in C57BL/6J-*Mcpt5*-Cre *ROSA26*-EYFP mice, we could not only confirm that uMCs accumulate during the receptive phase of the female (estrus, Figs 2A and 4) and their number decline at diestrus (Figs 2B and 4), but also show their density and localization. In previous studies, we could detect uMCs in longitudinally cut uterine sections distributed inter-implantational and in close proximity to the developing fetus (Woidacki *et al.* 2013). In this study, we observed that uMCs were additionally distributed in large numbers at the mesometrial site of the uterus that is in close proximity to the *decidua basalis* (Fig. 1D).

uMCs are abundant during early pregnancy and their frequency declines as gestation advances

To analyze the presence, distribution and density of uMCs during pregnancy, we concentrated first on days 0, 2 and 5 of the pre- and peri-implantation phase and later on days 8, 10 and 14 of the post-implantation phase. Uterine tissues at all analyzed pregnancy stages are characterized by remarkably large numbers of *Mcpt5*-expressing MCs (Figs 3A, B, C, D, E, F and 4). During the pre-implantation phase (days 0 and 2) when the uterus is preparing for the nidation of the blastocyst (Wang & Dey 2006), an accumulation of uMCs at the mesometrial uterine site was evident (Figs 3A, B and 4). During the implantation process at gestational day 5, when the blastocyst is in close physical and physiological contact

Figure 2 Uterine MCs (uMCs) accumulate at the receptive phase of the female – visualized live by applying intravital two-photon microscopy to C57BL/6J-*Mcpt5*-Cre *ROSA26*-EYFP mice. uMCs were monitored in virgin C57BL/6J-*Mcpt5*-Cre *ROSA26*-EYFP females at two phases of the estrous cycle, namely at estrus (A), which represents the receptive phase of the female and at diestrus (B) and EYFP signal was detected by emission at a wavelength of 500–550 nm. Blood flow was visualized by i.v. injection of QTracker 655 nontargeted QDots and detected by emission at a wavelength of 640–710 nm. Collagen fibers (gray) were detected by its second-harmonic generation (SHG) signal below 480 nm. Images were constructed by 3D rendering of z-stacks.

Figure 3 Uterine MCs were monitored in allogeneically, BALB/c mated C57BL/6J-Mcpt5-Cre ROSA26-EYFP females at gestational days 0 (A, depth 75 μ m), 2 (B, depth 81 μ m), 5 (C, depth 126 μ m), 8 (D, depth 111 μ m), 10 (E, depth 51 μ m) and 14 (F, depth 84 μ m) and EYFP signal was detected by emission at 500–550 nm. Blood flow was visualized by i.v. injection of QTracker 655 nontargeted QDots and detected by emission at 640–710 nm. Collagen fibers (gray) were detected by its second-harmonic generation (SHG) signal below 480 nm.

to the uterine endometrium (Dey *et al.* 2004), uMCs were present in comparable numbers as in non-pregnant uteri at estrus albeit this did not reach significance levels when compared with diestrus (Figs 3C and 4). Around day 8 of gestation when placentation starts (Rugh 1990) and spiral arteries appear histologically within the decidua basalis (Burke *et al.* 2010), uMCs are present (Fig. 3D) in slightly lower numbers than at early pregnancy (Fig. 4). At gestational day 10 when the placenta and the fetus are simultaneously growing, uMCs could be still detected mesometrially but their number was significantly lower than at estrus (Figs 3E and 4). At mid-gestation (day 14 of pregnancy) when the main focus is on fetal rather than on placental development (Suzuki *et al.* 1997), uMCs are present but in significantly lower numbers when compared with estrus (Figs 3F and 4).

Discussion

The *in vivo* adaptation of two-photon microscopy for monitoring MC distribution at the fetal–maternal interface offers a novel, impressive and powerful approach to address the evident relevance of uMCs to reproductive processes. The application of two-photon microscopy to transgenic mouse models increases exponentially (Zipfel *et al.* 2003). Of interest for the reproduction field, it has been recently reported that this methodology can be used for the tracking of dendritic cells (DCs; Zenclussen *et al.* 2013) and regulatory T cells (Treg; Teles *et al.* 2013) as well as for the imaging of the mouse placenta under physiological (Zenclussen *et al.* 2012) as well as pathophysiological conditions (De Moraes *et al.* 2013).

The generation of new mouse models whose MC deficiency is independent of restricted *Kit* gene expression (Müsch *et al.* 2008, Dudeck *et al.* 2011, Feyerabend *et al.* 2011) initiated a continuing controversy about the scientific significance of the *Kit*-dependent

models (Katz & Austen 2011, Brown *et al.* 2012) which were used for the investigation of MC biology *in vivo* during the last decades. The application of intravital microscopy to C57BL/6J-Mcpt5-Cre ROSA26-EYFP mice in which Mcpt5-producing MCs are detectable by the expression of EYFP (Scholten *et al.* 2008) represents a useful tool to further address the *Kit*-independent action of MCs and is of special interest for us.

Figure 4 Intravital two-photon microscopy allows the quantification of uterine MCs (uMCs) in virgin and pregnant uteri of C57BL/6J-Mcpt5-Cre ROSA26-EYFP mice. The number of MCs was determined in representative z-stacks of virgin or pregnant female C57BL/6J-Mcpt5-Cre ROSA26-EYFP mice ($n=5$ /cycle in non-pregnant and $n=3$ /stage in pregnant mice) using Zeiss LSM Image Browser software. Significantly higher numbers of uMCs were counted at estrus (receptive phase) in comparison to diestrus of the estrous cycle. At early pregnancy stages, the number of uMCs remained high and comparable to the values observed in estrus but declined as pregnancy advanced. Data were presented as mean $\pm$ S.E.M. and differences were calculated using ANOVA test and Tukey's multiple comparison test *post hoc*, * $P<0.05$ and ** $P<0.005$. GD, gestational day.

We adapted the technique introduced by [Zenclussen *et al.* \(2013\)](#) to the specific area of the uterus in which MCs are mainly distributed. Besides, we also investigated pregnant uteri that constituted a special challenge because of the increasing contractions as pregnancy advances. Herein, we report that imaging of the uterus was possible using a custom-made holder without any further manipulation of the tissue. MCs accumulated at the receptive phase of the female, namely in estrus when the uterine tissue is prepared for the nidation of the blastocyst. A similar distribution pattern could be observed live for DCs which additionally showed a clustering in estrus ([Zenclussen *et al.* 2013](#)) and are known to be crucial for the implantation process ([Plaks *et al.* 2008](#)). The accumulation of MCs at estrus could affect the physical as well as physiological contacts between the blastocyst and the uterine epithelium. In this study, a remarkably large number of uMCs at the mesometrial site of the virgin and pregnant uteri at all pregnancy stages were located in close proximity to blood vessels. To date, MC distribution pattern in uterus was exclusively referred to histological dying techniques applied to fixed tissue ([Gaytan *et al.* 1991](#), [Karaca *et al.* 2008](#)). In this study, we demonstrate that the number of MCs in the uterus is much higher as it was suspected. We can further confirm their localization close to vessels; this may be beneficial for the release of MC-specific mediators into the vasculature. Moreover, the immediate vicinity of MCs located at the mesometrial region to the fetoplacental unit suggests an interaction with other immune cells such as uterine NK cells, which are located within the mesometrial lymphoid aggregate of pregnancy (MLap) as well as within the decidua basalis ([Croy *et al.* 2006](#)). This together with the observation that uMCs are additionally located inter-implantational ([Woidacki *et al.* 2013](#)) suggests a significant role of uMCs and MC-specific mediators in reproductive processes such as implantation, placentation and later spiral artery remodeling. Recently published data point out that their absence is associated with abnormal implantation, aberrant placentation and inadequate spiral artery remodeling ([Woidacki *et al.* 2013](#)).

During the pre-implantation phase, the uterine tissue undergoes tremendous remodeling processes as a reaction to the invasion of fetal-derived trophoblasts ([Rugh 1990](#)). MCs are best known for their broad array of granule-stored mediators. The majority of these mediators are directly or indirectly involved in tissue remodeling and angiogenic processes including matrix metalloproteases, tryptases, chymases, tPA, TGF β , fibroblast growth factor and VEGF ([Galli *et al.* 2005](#)). During the implantation process when the trophoblast cells penetrate the uterine epithelium and decidualization starts, we found uMCs to be present and abundant at the mesometrial area of the uterus. In mice, the inner cell mass of the blastocysts is oriented mesometrially ([Dey *et al.* 2004](#)). The remarkably large amount of uMCs at

the mesometrial site of the uterus before and during implantation strongly suggests that they exert a positive influence on the establishment of pregnancy. However, and in contrast to this concept, in 1996 a significant role for MCs in the process of implantation was excluded. This conclusion was based on a negligible amount of MCs found in uterine tissue ([Salamonsen *et al.* 1996](#)). Back then, the quantification of MCs was only possible *ex vivo* in fixed tissue by means of immunohistochemical techniques. The introduced visualization of numerous uMCs live and *in vivo* using intravital microscopy suggests that inadequate fixation procedures of the tissue could cause false results as suggested by [El Sayed & Dyson \(1993\)](#). We did not perform confocal microscopy of fixed or unfixed tissue of C57BL/6J-*Mcpt5-Cre ROSA26-EYFP* mice to state whether this alternative method yields similar results as ours in *in vivo* observations. It is, however, evident from this study that MCs are present in significant numbers and at strategic sites at the fetal-maternal interface, which supports recent data on their important role for pregnancy establishment ([Woidacki *et al.* 2013](#)).

During the post-implantation phase, when placentation starts (day 8) ([Rugh 1990](#)) and spiral arteries appear within the decidua basalis ([Burke *et al.* 2010](#)), uMCs remained present in the uterus. Recently, we demonstrated that mice deficient in uMCs showed decreased placental surface areas and an inadequate remodeling of spiral arteries compared with WT C57BL/6J mice ([Woidacki *et al.* 2013](#)), whose uterine tissue is characterized by the presence of MCs at days 8 and 10 of pregnancy as shown in the present paper. Defective placental development as well as spiral artery formation cause fetal growth retardation ([Ashkar *et al.* 2000](#), [Song *et al.* 2002](#), [Watson & Cross 2005](#)) as both structures are crucial for maternal and fetal blood exchange, and therefore, for supplying the fetus with oxygen and nutrients. Even at day 14 of pregnancy when the fetus but not the placenta needs to grow ([Suzuki *et al.* 1997](#)), the amount of uMCs is still considerable although significantly lower when compared with that at estrus. These observations are indicative of the involvement of uMCs in pregnancy establishment rather than its maintenance. This may be due to the secretion of soluble factors contained in their granules. One important mediator was found to be Gal1. MCs deficient in *Lgals1* were not efficient in restoring placental growth as MCs from WT animals did when transferred into *Kit*-deficient mice ([Woidacki *et al.* 2013](#)). It is, however, possible that other uMC mediators are involved in this late process, too. Moreover, based on our data ([Woidacki *et al.* 2013](#)), we speculated that the mucosal MC phenotype that is also present in uterine tissue plays a role in mediating pre-, peri- and post-implantation events. Thus, the unique uterine microenvironment during pregnancy might influence the distribution/expression balance between both the MC phenotypes. The detection of uMCs that undergo a

transdifferentiation process (Woidacki *et al.* 2013) might support this assumption.

Although the detected EYFP-positive cells represent connective tissue-type MCs based on the expression of the serine protease *Mcpt-5* (Dudeck *et al.* 2011) and uMCs display a unique population composed of mucosal, connective tissue-type and a third transdifferentiation cell-type MCs (Spicer 1960, Woidacki *et al.* 2013), C57BL/6J-*Mcpt5-Cre ROSA26-EYFP* mice are a valuable and reliable model for studying uMC behavior live and *in vivo*. To the best of our knowledge, except one alternative model that is based on a cross of a *ROSA26-tdRFP* reporter strain (Luche *et al.* 2007) with the *Mcpt5-Cre* transgenic mouse strain (C57BL/6J-*Mcpt5-Cre Rosa26-tdRFP*) (Roers A 2012, personal communication), no other MC-specific reporter strains are available.

During the imaging process, uterine contractions, which would have negatively influenced the quality of pictures and movies, were reduced to a minimum by applying caffeine-soaked cotton rolls that ensured a permanent drenching of the uterus. Former problems of intravital technology concerning the amenability of the organ could be resolved by performing handling modifications of the animal and uterus respectively. According to the applied imaging procedures and a custom-made microscope stage, intravital images of placenta (Zenclussen *et al.* 2012) and uterus (Zenclussen *et al.* 2013) emerged as an adequate appliance for the basic fixation of the uterus for analyzing uMCs. A self-made fixator composed of tear-resistant aluminum foil served as a fine fixation for the uterus. The addition of low-melting agarose allowed a constant and long-lasting moistening of the focal area.

The application of two-photon microscopy to C57BL/6J-*Mcpt5-Cre ROSA26-EYFP* mice represents a novel and exciting approach to track MC behavior *in vivo*. It could be a powerful tool to bring to light the so-far controversial discussed influence of MCs on reproductive processes and pregnancy. We do hope that the introduced visualization of uMCs might help in the future for addressing elementary questions regarding MCs and their participation in physiological and pathological processes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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