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1 **Luteal angiogenesis and its control.**

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Abstract

Angiogenesis, the formation of new blood vessels from pre-existing ones, is critical to luteal structure and function; In addition, it is a complex and tightly regulated process. Not only does rapid and extensive angiogenesis occur to provide the corpus luteum (CL) with an unusually high blood flow and support its high metabolic rate, but in the absence of pregnancy the luteal vasculature must rapidly regress to enable the next cycle of ovarian activity. This review describes a number of the key endogenous stimulatory and inhibitory factors, which act in a delicate balance to regulate luteal angiogenesis and ultimately luteal function. *In vitro* luteal angiogenesis cultures have demonstrated critical roles for fibroblast growth factor 2 (FGF2) in endothelial cell proliferation and sprouting, whilst other factors such as vascular endothelial growth factor (VEGFA) and platelet derived growth factor (PDGF) were important modulators in the control of luteal angiogenesis. Post-transcriptional regulation by small non-coding micro-RNAs, is also likely to play a central role in the regulation of luteal angiogenesis. Appropriate luteal angiogenesis requires the coordinated activity of numerous factors expressed by several cell types at different times and this review will also describe the role of perivascular pericytes and the importance of vascular maturation and stability. It is hoped that a better understanding of the critical processes underlying the transition from follicle to CL, and subsequent luteal development will benefit the management of luteal function in the future.

Key words: ovary, corpus luteum; angiogenesis; vasculature; FGF2, VEGFA

1. The importance of luteal angiogenesis

The corpus luteum (CL) is a transient endocrine structure that is critical for the establishment and maintenance of pregnancy in mammals. It is formed from the remnants of the ruptured follicle post-ovulation and undergoes remarkable growth, differentiation and remodelling. Often compared to fast growing tumours, the dramatic growth of the CL is reliant upon angiogenesis, or the formation of new blood vessels from pre-existing vessels from the follicular theca layer [1].

The crucial importance of angiogenesis to luteal structure and function has been demonstrated in a number of species, including domestic ruminants. For example, the experimental blockade of angiogenesis resulted in reduced CL number, limited luteal vasculature and marked inhibition of steroidogenesis in rats [2]. Similarly, intra-follicular or systemic administration of anti-angiogenic factors (e.g. VEGFA trap) to non-human primates altered ovulation, reduced endothelial cell proliferation in the CL and inhibited progesterone production [3, 4]. Furthermore, intra-luteal anti-angiogenic treatments reduced CL volume and plasma progesterone concentrations and disrupted normal luteal gene expression in the cow [5].

Transgenic mouse models which have targeted angiogenic signals have similarly resulted in both diminished ovarian vasculature and fertility [6]. In addition, poor vascularisation has been linked to inadequate luteal function, such as that observed following ovulation induction in women and livestock, and in the peripubertal and postpartum periods in domestic animals [7-9].

2. Establishment of the luteal vasculature

Luteal angiogenesis originates from the developing follicle. Early follicles (primordial and primary) have no established vascular supply of their own. Rather, blood vessels develop as follicles undergo continued growth, with endothelial cell recruitment occurring from the ovarian stromal compartment. Follicular vessels remain within the thecal layer and are excluded from the granulosa cell layer by the basement membrane which divides the two. Following the luteinising hormone (LH) surge, the breakdown of the basement membrane enables blood vessels to invade the granulosa layer as cellular remodelling begins [10]. The continuation of development from ovulatory follicle to corpus luteum therefore also suggests that appropriate follicular development, including blood vessel formation, may be critical to the success of subsequent luteinisation [11]. Indeed, recent evidence showed that follicular vascularity is positively correlated with luteal blood flow and progesterone production [12]. Furthermore, the degree of follicular vascularisation has been associated positively with follicular dominance and negatively with atresia [13].

The early events of luteinisation are accompanied by marked cell proliferation, with proliferation indices around 40% [14, 15]. Critically, the majority of mitotic cells are not steroidogenic luteal cells, but rather they are from the microvasculature [14, 15]. Indeed, endothelial cells are a prominent cell-type within the corpus luteum, occupying around 15% of luteal tissue volume, and representing around 50% of all cells at mid-cycle [16]. Such an extensive contribution to the mature luteal tissue ensures that nearly all steroidogenic cells are in immediate contact with at least one capillary [17].

3. The response to LH

Ovulation and early luteinisation are characterised by complex changes in gene expression, with perhaps hundreds of genes differentially expressed [18-20]. The molecular response to an ovulatory dose of LH is also rapid, with the first changes in gene expression occurring within 30 minutes. Genes associated with ovulation have been implicated in inflammation, steroid and prostanoid pathways, proteolytic disruption of the tissue matrix and protection against oxidative stress [18]. Others have demonstrated that luteinisation is accompanied by a switch from a molecular signature of proliferation and metabolism to one where cell migration and angiogenesis predominate [19].

More recently, the potential importance of post-transcriptional regulation has come to the fore [21]. Small non-coding RNAs such as microRNAs (miRNAs) function primarily as negative regulators of gene expression and are now thought to be key regulators of ovarian function, including the follicular-luteal transition [22]. Mice deficient for the miRNA processing enzyme Dicer displayed luteal insufficiency that was associated with poor angiogenesis and reduced luteal vascular density [23]. In the sheep ovary, a total of 17 miRNAs were identified whose abundance varied significantly between follicular and luteal phases and are potential important regulators of luteinisation [24]. This included decreased levels of miR-503 (a known angiogenesis inhibitor) during early luteinisation. Interestingly, the theca cell layer was the major site of miRNA expression, with vascular components of the thecal layer expected to be key targets of miRNA regulation, as has been shown in other tissues [25].

4. Control of luteal angiogenesis

4.1. Stimulatory factors

Follicular fluid accumulates angiogenic factors that are likely to provide an initial stimulus to post-ovulatory angiogenesis [26]. Corpora lutea subsequently produce pro-angiogenic factors throughout the luteal phase and into pregnancy [27, 28] and their actions can result in endothelial cell proliferation, migration and tubule formation *in vitro* and *in vivo.* A significant number of factors are mediators of angiogenesis [29], including vascularisation of the CL. Key amongst these are the heparin-binding factors, namely vascular endothelial growth factor A (VEGFA) and fibroblast growth factor 2 (FGF2).

VEGFA is a potent endothelial mitogen, which exists as one of several isoforms [30] as a result of the alternative splicing of a single VEGFA gene. $VEGFA_{165}$ is the predominant (human) protein isoform produced by a variety of cells and is so named due to its 165 amino acids, following cleavage of the signal sequence. Molecular species with 121, 189, and 206 amino acids are also described, plus several rare species such as $VEGFA_{145}$ and $VEGFA_{183}$; in the cow, each isoform is one amino acid shorter [31]. All isoforms contain domains that enable receptor binding and all are biologically active. The various isoforms do exhibit different biochemical properties however, with some predominantly soluble species, such as $VEGFA_{121}$ and $VEGFA_{165}$ and others ($VEGFA_{189}$ and $VEGFA_{206}$) significantly cell or matrix-bound until released following proteolysis of the ECM [30].

The presence of *VEGFA* mRNA and protein has been demonstrated in the ovary of many species [32-36]. In bovine antral follicles, VEGFA was localised to granulosa cells, and cells of the theca layer, and increased with follicular growth and development (Figure 1; [36]). In the bovine CL, *VEGFA* mRNA was detected throughout the luteal phase and during pregnancy,

but decreased in the late luteal phase [37], and luteal steroidogenic cells were the major cellular site of VEGFA protein expression (Figure 1; [37]).

Members of the VEGF family interact with several receptors and co-receptors to exert their actions [30]. VEGFA binds to the related receptors VEGFR1 (Flt1) and VEGFR2 (KDR or Flk1), with the mitogenic and angiogenic responses to VEGFA largely mediated via VEGFR2. These tyrosine kinase receptors are expressed on the surface of endothelial cells, including those of the ovary [38]. Indeed, VEGFR1 and R2 were expressed by microvascular endothelial cells derived from the bovine CL [38]. Others have detected VEGFR2 in bovine luteal cells and smooth muscle cells, as well as endothelial cells by immunohistochemistry [39]. VEGFR1 expression did not vary according to luteal stage, whilst VEGFR2 mRNA was most highly expressed in the early [37] to mid [39] CL.

As eluded to earlier, neutralisation of VEGF by several routes and in several species including the cow, caused marked reductions in luteal vascularisation and progesterone production [4, 5, 40]. In addition to its ability to stimulate an angiogenic response, VEGFA infusion also stimulated progesterone production by micro-dialysed bovine CL *in vitro* [41]. VEGFA is therefore considered essential to luteal structure and function.

Fibroblast growth factor 2 is also a prominent regulator of luteal angiogenesis. Often overlooked in importance relative to VEGFA, early work in the sheep and cow demonstrated the significant inhibition of endothelial mitogenic activity following immuno-neutralisation of FGF2 from luteal-conditioned media [28, 42]. In addition, the neutralisation of FGF2 *in vivo* resulted in luteal disruption, with CL volume, steroidogenic function and gene expression significantly diminished [5]. Indeed, in the cow, luteal FGF2 concentrations have been shown to be more dynamic around the follicular-luteal transition than VEGFA,

suggestive of a more critical role for FGF2 in early luteinisation [26]. FGF2 protein levels were significantly elevated in early bovine CL (day 1-2) and declined with subsequent luteal development [26].

In order to further dissect the regulation of luteal angiogenesis in the cow, we established a novel, physiologically relevant *in vitro* culture system that mimics both luteal steroidogenic function and angiogenesis [43]. The use of luteal endothelial cells is critical, since endothelial cells and angiogenic responses are known to differ between tissues and microenvironments [44]. In the bovine luteal angiogenesis culture system early CL (days 1-4) are dissected from the ovary, dissociated enzymatically and mixed luteal cells (including steroidogenic, endothelial (EC), fibroblast and perivascular cells) are then plated on fibronectin-coated wells. Luteal cells are grown in culture for up to 9 days, in the presence of endothelial-specific media, plus or minus angiogenic support (VEGFA and/or FGF2). During this time, steroidogenic cells produce progesterone in a LH-responsive manner [43]. In addition, endothelial cells can be stimulated to undergo characteristic tubule-like growth, resulting in the formation of complex and extensive highly-branched EC networks (Figure 2).

Using this system, the degree of bovine EC network formation was stimulated by both VEGFA and FGF2 [43, 45]. FGFR1 inhibition, via treatment with SU5402 throughout the culture period, dramatically reduced the total area of EC networks by 94% versus controls, as a result of reductions in the number of individual EC networks and a tendency to reduce the size of each network [45]. In contrast, the inhibition of VEGFR2 signalling was more modest, reducing EC area by around 60% [45]. Strikingly, the response to FGFR1 inhibition was observed despite the presence of VEGFA treatments, further supporting the critical role of FGF2 in luteal endothelial network formation [45, 46].

FGFR1 inhibition was further utilised to elucidate which stage(s) of luteal angiogenesis are most dependent upon FGF2 stimulation [46]. In particular, the earliest stages of angiogenesis were most sensitive to FGFR1 inhibition *in vitro*, as evidenced by a 64% reduction in total EC networks following SU5402 treatment on days 0-3 and by around 81% following treatment on days 3-6 versus controls. Days 3-6 in culture is a period of intense reorganisation, when EC begin to sprout from EC islands and form tubule-like structures (Figure 2). Further analysis revealed that FGFR1 inhibition on days 3-6 resulted in a marked reduction in EC network branch points. This suggested a critical role for FGF2 in endothelial sprouting, as well as endothelial cell proliferation [46]. Indeed, FGF2 has been shown to promote vascular branching in several systems: FGF2 increased the density and branching of the microvessels of the chorioallantoic membrane [47] and transgenic mice with disrupted FGFR1 signalling displayed retinal phenotypes with reduced capillary density and branching [48]. Furthermore, FGF2 dose-dependently increased the degree of EC branching in a bovine luteinising follicular culture system [49].

Endothelial cell sprouting is a complex process, involving coordinated cell migration and phenotypic specialization in response to directional cues [50]. Early in the process, certain endothelial cells emerge as tip cells, characterised by numerous cellular extensions or filopodia which sense the surrounding environment, whilst others remain at the stalks of the vascular sprouts. Recent data suggesting that FGFR1 participates in tip cell function and filopodia formation via interactions with the cytoskeletal protein Nostrin, which is involved in membrane dynamics, further supports a role for FGF2 in EC sprouting [51].

Hypoxia is potent stimulator of angiogenesis in numerous situations. There is evidence that hypoxic conditions found in the early CL might upregulate FGF2 expression and hence

stimulate luteal angiogenesis [52]. Protein levels of the hypoxia-induced transcription factor, HIF1A were greatest in the early bovine CL and decreased thereafter [53] in a pattern similar to FGF2 [54]. Furthermore, in human umbilical vein endothelial cells in response to hypoxia, upregulation of HIF1A resulted in branching morphogenesis via induction of FGF2 [55]. Positive feedback between HIF1A and FGF2 has also been reported [52].

The role of FGF2 in regulating both luteal angiogenesis and the production of the maternal recognition signal, interferon tau, in ruminants [56] has made it a valid candidate gene in the search for genetic markers associated with reproductive efficiency. The association between FGF2 gene variants (single nucleotide polymorphisms; SNP) and a range of fertility-related traits has been investigated in several studies. In Holstein cows, embryo survival *in vitro* was significantly associated with the genotype of the intronic SNP11646 (rs110937773; FGF2 intron 1) [57]. However, no significant associations were observed between this or another polymorphism (SNP23; rs208883803) and a range of fertility traits, including those potentially influenced by luteal function or cyclicity such as number of inseminations per conception, calving to conception interval or conception rate [58] and Woad et al, unpublished.

The delta-Notch signalling pathway has been implicated in the determination of cell fate and patterning associated with endothelial cell sprouting and vascular development [59, 60]. Endothelial cells express Notch receptors and their ligands (Delta-like ligand 1, 4 and Jagged1), and the balance of signalling determines tip versus stalk cell fate, with tip cells preferentially expressing Dll4, whilst stalk cells express predominantly Jagged1. Notch receptors and ligands have been localised to the follicular and luteal vasculature [61-63], including in the bovine CL (Robinson et al., unpublished). In the marmoset, inhibition of Dll4

in the periovulatory period, led to increased angiogenesis in the early luteal phase. However, the vasculature that was formed was dysregulated and non-functional, resulting in decreased progesterone production [64]. In the bovine luteal angiogenesis culture system, the addition of γ-secretase inhibitors to block Notch signalling reduced EC network formation (Robinson et al., unpublished).

4.2. Inhibitory factors

The control of angiogenesis requires the appropriate balance of both positive and negative signals. In the follicle, angiogenesis is thought to be actively restrained until after ovulation, thus preventing premature vascularisation. Pigment epithelium derived factor (PEDF) is a recently described, physiological inhibitor of angiogenesis, with ovarian influence [65]. PEDF is secreted by mouse and human granulosa cells and this secretion was sharply decreased by progesterone treatment. It is inhibitory in angiogenesis assays, and the loss of PEDF led to accelerated angiogenesis *in vitro*.

Thrombospondins (THBS1 and 2) are anti-angiogenic factors with many mechanisms of inhibition. THBS1 can bind FGF2 and this sequestration [66] then inhibits its pro-angiogenic actions. The modulation of angiogenesis by thrombospondins may be particularly important for luteal regression. For example, in the sheep CL, THBS1 expression was upregulated during luteolysis [67]. Furthermore, prostaglandin PGF2α-induced increased expression of THBS1 and 2, and their receptor CD36 in the bovine CL in a stage dependant manner, with the PG-refractory CL showing no upregulation [68]. Critically, luteal angiogenesis was inhibited in response to these factors even in the presence of pro-angiogenic FGF2, and inhibition was also observed in the absence of FGF2 [68].

Alterations in angiogenic support (VEGFA and FGF2), increased angiogenic inhibition (thrombospondins, pentraxin 3 and transforming growth factor B1) and changes in luteal blood flow (endothelin-1 and nitric oxide) can all occur in response to luteolytic-PG and will contribute to the eventual disruption of the microvasculature and subsequent luteal regression [68-71].

The VEGFA system has anti-angiogenic components, several of which have been implicated in ovarian function. The complex splicing of the *VEGFA* mRNA, leads to the production of an alternative family of VEGFAxxxb isoforms (where xxx refers to the number of amino acids), which are generated by distal splice site utilisation in exon 8 [72]. In the porcine CL, VEGFA164b was detected at low levels throughout the luteal phase [73]. In the marmoset monkey, VEGFA165b comprised around 65% of total VEGFA in CL-bearing ovaries [6]. Overexpression of VEGFA165b in mice reduced follicular development, leading to lower CL number, reduced luteal size and decreased microvascular density and stability [6]. In contrast, VEGFA164b mRNA was not detected in the CL at any stage in the cow [74].

VEGFA activity can be further modified by soluble receptor isoforms, sVEGFR1 and sVEGFR2, which act as VEGF binding proteins. In the bovine CL, soluble receptors were found throughout the luteal phase. Indeed, sVEGFR1 was more than 100 times as abundant as the membrane bound receptor [74]. The functional significance of this observation remains to be elucidated.

Vasohibin1 (VASH1) is a further factor with negative regulatory potential [75]. In the bovine CL, It is expressed primarily in the luteal endothelial cells, is induced by VEGFA and then inhibits VEGFA actions in a classical negative feedback mechanism. It was suggested that

VASH1 might act to fine tune pro-angiogenic signals and prevent inappropriate vascularisation [75]; however its exact role remains to be demonstrated.

5. Stabilising the vasculature

In addition to extensive and rapid vascularisation, the corpus luteum also requires newly formed vessels to undergo maturation and stabilisation in order to be fully functional. The angiopoietins (ANGPT1 and ANGPT2) have particular importance in vessel stability [29]. They are considered important partners for VEGFA and act primarily through the Tie2 receptor. ANGPT2 is an endogenous Tie2 antagonist [76], that results in vessel destabilisation and the ratio of ANGPT1/ANGPT2 is therefore considered of critical importance to vessel fate. During the follicular-luteal transition, the destabilising effect of ANGPT2 is thought to maintain vascular plasticity, hence modifying responsiveness to pro-angiogenic signals such as VEGFA [76].

Pericytes (perivascular mural cells) are important constituents of microvessels, with key roles in vascular development and function, including the stabilisation and maturation of vessels [77, 78]. The recruitment of pericytes to the blood vessel wall and their subsequent interactions with endothelial cells are critically regulated by platelet derived growth factor (PDGF) signalling via the PDGF receptor B (PDGFRB). The inhibition of pericyte-recruitment around ovulation reduced the number of luteal structures formed in rodents [79, 80] and induced widespread luteal haemorrhage, suggestive of an obligatory requirement for pericyte involvement in appropriate luteal angiogenesis. Furthermore, inhibition of PDGF signalling *in vitro* reduced endothelial cell network formation in our bovine luteal angiogenesis culture system and the networks were most sensitive to receptor blockade during the early stages of angiogenesis [45]. Smooth muscle actin-positive mural cells

(putative pericytes) were found closely associated with endothelial cells *in vitro* (Figure 3; [49]). Mural cells were localised both as integral components of EC islands and on the borders of the islands. During the follicular-luteal transition in the cow, pericytes appeared to migrate ahead of EC from the theca layer into the luteinising granulosa cells. This was potentially to guide sprouting processes and/or lay down ECM such as fibronectin [10, 54]. Similarly, in culture, mural cells were found at the tips of endothelial sprouts (Figure 3; [49]). This might indicate that pericytes play an active role at all stages of luteal angiogenesis.

6. Conclusion

Angiogenesis is critical to the structure and function of the corpus luteum. It is a complex process that is under exquisite control, requiring the interaction of numerous factors and several cell types during a period of remarkable dynamism. Whilst there is good evidence for several critical regulators with both pro- and anti-angiogenic functions, further investigation of the mechanisms of luteal angiogenesis is essential for improving our understanding of luteal function

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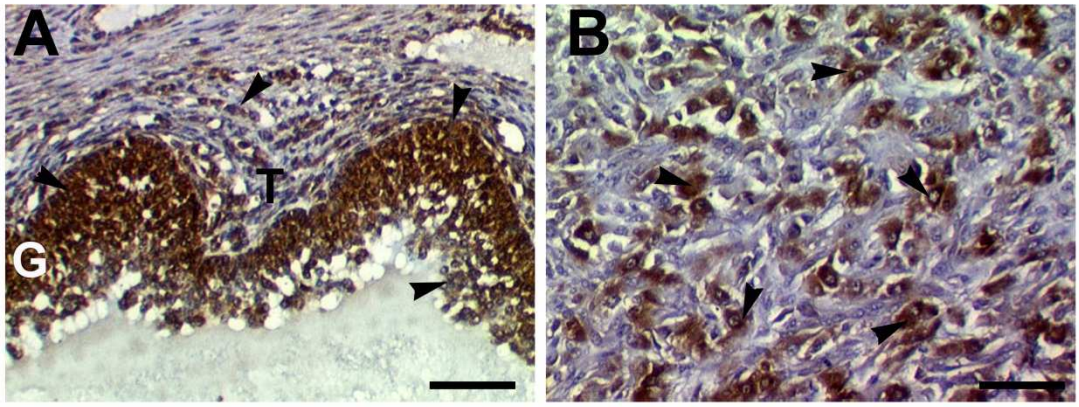
Figure 1: The localisation of VEGFA in (**A**) a dominant follicle and (**B**) the corpus luteum (CL) in the cow. There was intense staining of VEGFA in the granulosa (G) layer (brown staining, arrowhead). VEGFA was also present, albeit at much lower levels in the theca (T) layer. (B) shows the intense staining of VEGFA in the steroidogenic cells (arrowhead) of the day 5 CL. There was some variation in the stain intensity between different steroidogenic cells across the whole section. The scale bar represents 200µm. Robinson et al., unpublished observations.

Figure 2: Time course of bovine luteal angiogenesis *in vitro*, showing a bovine ovary bearing (**A**) an early corpus luteum (arrow) selected for culture, and subsequent endothelial cell growth between 12 h and 9 days. Endothelial cells were immuno-localised by von Willebrand factor staining (brown) after (**B**) 12 and (**C**) 18 h (day 1) and then every 24 h; day 2 (**D** and **E**), day 3 (**F**), day 4 (**G**), day 5 (**H**), day 6 (**I**), day 7 (**J**), day 8 (**K**) and day 9 (**L**). Bar represents 100 µm. Adapted from [46].

Figure 3: The temporal-spatial interactions between endothelial cells and perivascular mural cell in the bovine luteal-endothelial co-culture system, on (**A**) day 6 or (**B**) day 9 of culture. The endothelial cells (EC) were immuno-stained with von Willebrand (green) while the mural cells were identified by smooth muscle actin immunohistochemistry (red). The nucleus was counterstained with DAPI (blue). On day 6, the EC were present in large islands of cells containing several hundred cells. Inter-dispersed within these EC islands and around the edge were mural cells. On day 9, the EC had a much more network-like appearance with multiple sprouts projecting away from the centre of the EC island. Again, mural cells were often closely associated with the EC networks. On both days 6 and 9, mural cells were often present at the tips of sprouting EC (arrowhead). The scale bar represents 200µm. Robinson, Woad et al., unpublished observations.



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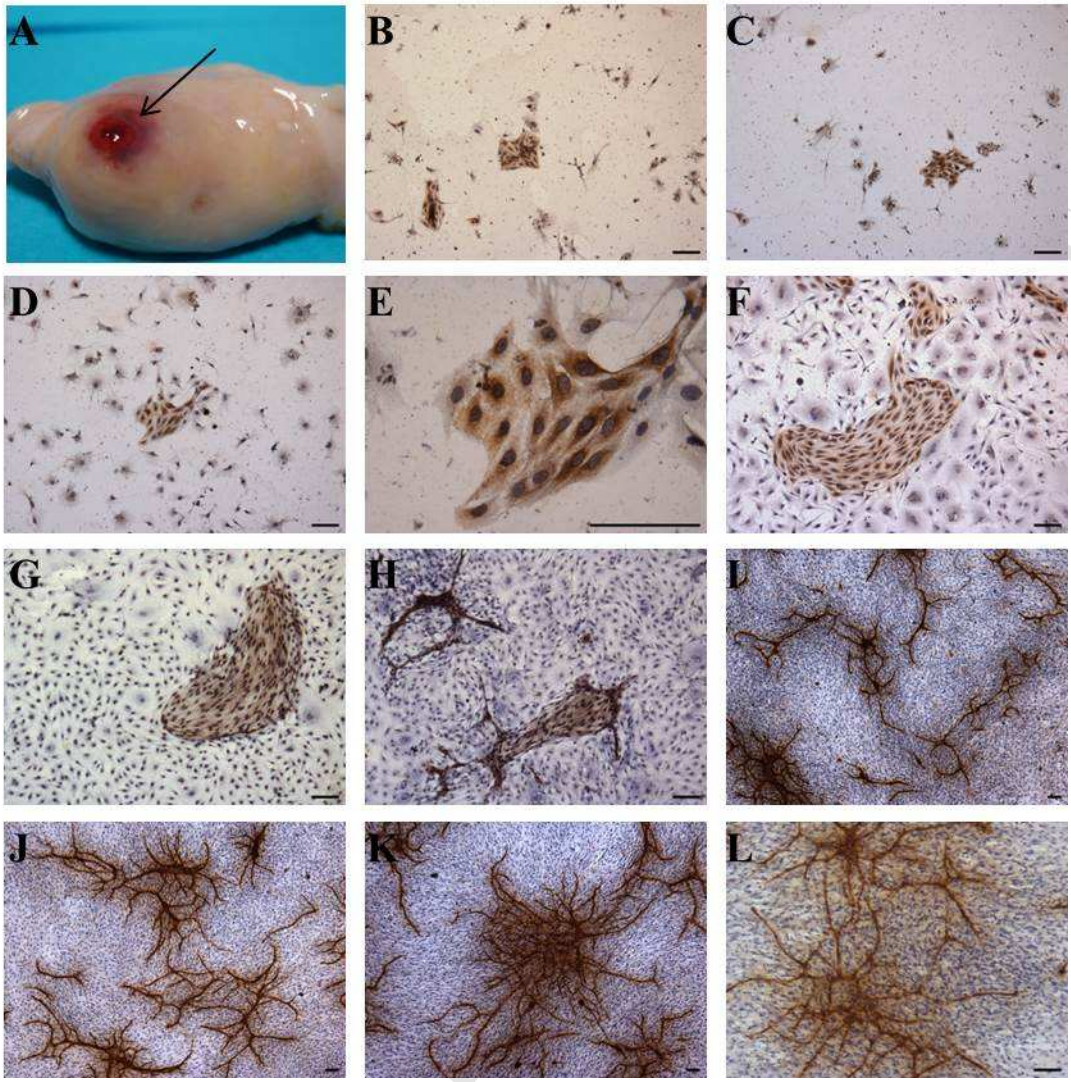
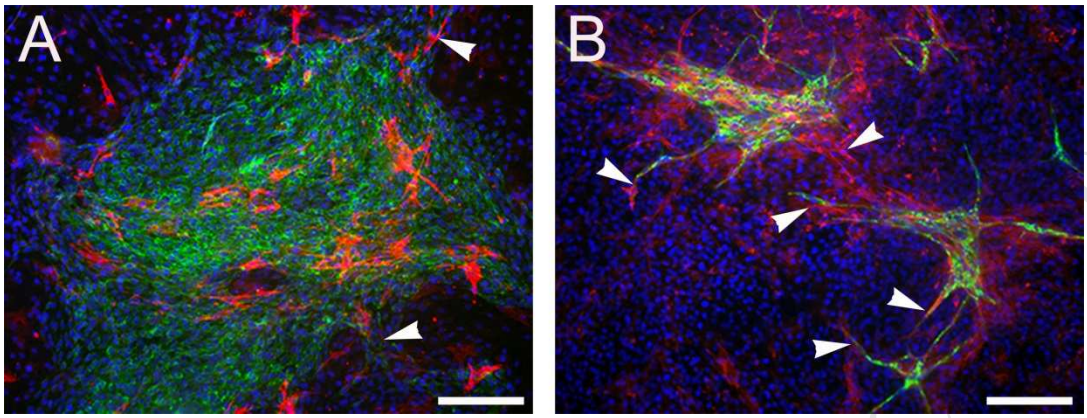


Figure 2:



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