Uterine natural killer cells mediate dedifferentiation of spiral artery vascular smooth muscle cells in early human pregnancy

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Running title: Uterine spiral artery muscle dedifferentiation

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Abstract

Study question: Is vascular smooth muscle cell (VSMC) dedifferentiation a feature of uterine spiral artery (SpA) remodelling in early human pregnancy?

Summary answer: Remodelling of human uterine SpAs is associated with dedifferentiation of VSMCs and can be induced in vitro by uterine natural killer (uNK) cells and extravillous trophoblast cells (EVTs).

What is known already: Uterine SpAs undergo profound morphological changes in normal pregnancy with replacement of the musculoelastic arterial wall structure by fibrinoid containing EVTs. The fate of VSMCs in SpA remodelling is unknown; in guinea pig uterine artery VSMCs dedifferentiate, remain in the vessel wall and differentiate after parturition to restore the arterial wall. There is increasing evidence that uNK cells play a role in SpA remodelling. We hypothesised that SpA remodelling in human pregnancy is associated with VSMC dedifferentiation, initiated by uNK cell derived growth factors.

Study design, size, duration: Formalin fixed, paraffin embedded placental bed biopsies were immunostained for angiogenic growth factor (AGF) receptors and markers of VSMC differentiation. An in vitro model of SpA remodelling using chorionic plate arteries (CPAs) was used to test the effect of different cell types and AGFs on VSMC differentiation.

Participants/materials, setting, methods: Placental bed biopsies were immunostained for vascular endothelial growth factor receptors 1-3 (VEGF-R1, VEGF-R2, VEGF-R3), transforming growth factor beta 1 receptors I and II (TGF-βRI, TGF-βRII), interferon gamma receptors 1 and 2 (IFN-γR1, IFN-γR2), Tie2, α-smooth muscle actin (α-SMA), H-caldesmon (H-Cal), myosin heavy chain (MyHC), osteopontin and smoothelin. Staining intensity was assessed using a modified quickscore. Expression

by VSMCs of the AGF receptors was confirmed by laser capture microdissection and real time RT-PCR of non-remodelled SpAs, after laser removal of the endothelium. As an in vitro model, VSMC differentiation was assessed in CPAs by immunohistochemistry after culture in uNK cell conditioned medium (CM), EVT-CM, uNK cell/EVT co-culture CM, Ang-1, Ang-2, IFN-γ, VEGF-A and VEGF-C, and after blocking of both Ang-1 and Ang-2 in uNK-CM.

Main results and the role of chance: SpA VSMC expression of Tie-2 (P=0.0007), VEGF-R2 (P=0.005) and osteopontin (P=0.0001) increased in partially remodelled SpAs compared with non-remodelled SpAs, while expression of contractile VSMC markers was reduced (α-SMA P<0.0001, H-Cal P=0.03, MyHC P=0.03, smoothelin P=0.0001). In the in vitro CPA model, supernatants from purified uNK cell (H-Cal P<0.0001, MyHC P=0.03, α-SMA P=0.02, osteopontin P=0.03), EVT (H-Cal P=0.0006, MyHC P=0.02, osteopontin P=0.01) and uNK cell/EVT co-cultures (H-Cal P=0.001, MyHC P=0.05, osteopontin P=0.02) at 12-14 weeks, but not 8-10 weeks, gestational age induced reduced expression of contractile VSMC markers, and increased osteopontin expression. Addition of exogenous (10ng/ml) Ang-1 (P=0.006) or Ang-2 (P=0.009) also reduced H-Cal expression in the CPA model. Inhibition of Ang-1 (P=0.0004) or Ang-2 (P=0.004) in uNK cell supernatants blocked the ability of uNK cell supernatants to reduce H-Cal expression.

Limitations, reasons for caution: This is an in vitro study and the role of uNK cells, Ang-1 and Ang-2 in SpA remodelling in vivo has not yet been shown.

Wider implications of the findings: VSMC dedifferentiation is a feature of early SpA remodelling and uNK cells and EVT play key roles in this process by secretion of Ang-1 and Ang-2. This is one of the first studies to suggest a direct role for Ang-1 and Ang-2 in VSMC biology.

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Key Words: vascular smooth muscle cells; dedifferentiation; spiral arteries; uterine natural killer cells; vascular remodelling; human pregnancy

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Introduction

Uterine spiral arteries (SpA) in decidualised endometrium and inner myometrium undergo profound morphological changes in successful human pregnancy. Deficient SpA remodelling occurs in various pregnancy complications, including pre-eclampsia, fetal growth restriction, preterm labour and late miscarriage (Brosens et al., 2011). As well as direct fetal and maternal morbidity and mortality, these conditions may also impact on health in later life for mothers and surviving babies (Barker, 2007; Brown et al., 2013; Wu et al., 2017).

SpA remodelling is characterised by replacement of medial vascular smooth muscle cells (VSMCs), extracellular matrix and elastic lamina by amorphous fibrinoid material containing intramural extravillous trophoblast cells (EVTs) (Pijnenborg et al., 2006; Smith et al., 2016). EVTs are required for complete SpA remodelling but there is increasing evidence for a maternal-mediated 'priming' phase preceding EVT invasion: 'decidua-associated' or 'trophoblast-independent' remodelling (Pijnenborg et al., 2006). SpAs in early pregnancy show dilatation, endothelial cell vacuolisation and basophilia, and VSMC hyperplasia, vacuolisation, separation and disorganisation in the absence of EVTs (Craven et al., 1998; Kam et al., 1999; Pijnenborg et al., 2006).

CD56+ uterine natural killer (uNK) cells account for up to 70% of leucocytes in decidual stroma in the first trimester (Bulmer et al., 1991); their accumulation around SpAs suggests a role in remodelling (Smith et al., 2009; Lash et al., 2016). Furthermore, uNK cells from early pregnancy decidua produce a range of angiogenic growth factors (AGFs) including angiopoietin-1 (Ang-1), Ang-2, transforming growth factor beta 1 (TGF-β1), vascular endothelial growth factor-C (VEGF-C), interleukin 8 (IL-8) and

interferon gamma (IFN-γ) (Hanna et al., 2006; Lash et al., 2006), all of which can cause morphological changes to pre-existing vascular networks (Distler et al., 2003; Ferrara, 2004). Using an *in vitro* model of SpA remodelling we have demonstrated that uNK cells from early human pregnancy can induce morphological changes in VSMCs (Robson et al., 2012).

The cellular mechanisms that underlie medial VSMC loss in SpAs in human pregnancy are not fully understood. Apoptosis has been suggested (Smith et al., 2009), but appears to be a rare event within the SpA artery wall itself (Bulmer et al., 2012). However, VSMCs migrate away from the SpA wall into decidual stroma during remodelling where they then appear to undergo apoptosis (Bulmer et al., 2012; Lash et al., 2016). In guinea pig, uterine VSMCs exhibit polygonal morphology and loss of desmin and α-smooth muscle actin (α-SMA) in pregnancy, with restoration three days post-parturition, suggesting that uterine VSMCs dedifferentiate during artery remodelling (Nanaev et al., 1995, 2000).

VSMCs can switch from contractile to synthetic phenotype according to local environmental cues (McDonald and Owens, 2007). Contractile VSMCs are elongated, spindle shaped cells characterised by high expression of contractile marker proteins such as α-SMA, H-caldesmon (H-cal) and myosin heavy chain (MyHC), whereas synthetic VSMCs have low contractile marker expression, less elongated rhomboid morphology and are more motile (Rensen et al., 2007). Modifiers of VSMC phenotype *in vitro* include biochemical factors such as platelet derived growth factor (PDGF) and TGF-β1, extracellular matrix components and physical factors such as tensile stress (Rensen et al., 2007; Rzucidlo et al., 2007).

In view of reports of uterine artery VSMC dedifferentiation in guinea pig, VSMC motility in humans and high AGF secretion by uNK cells, we hypothesised that VSMCs undergo dedifferentiation during SpA remodelling, mediated by paracrine signalling through uNK cell derived factors. To test this hypothesis, we examined expression of VSMC differentiation markers in both non-remodelled SpAs and SpAs in early stages of remodelling in the absence of EVT and used an *in vitro* model to determine which uNK cell derived AGFs may initiate VSMC dedifferentiation.

Materials and Methods

Samples

Placental bed biopsies for immunohistochemical studies were obtained as previously described (Robson et al., 2002) after elective surgical termination of apparently normal pregnancy at 8-14 weeks gestational age (n=15). Placenta and decidua for uNK cell and EVT cultures were obtained at 8-10 and 12-14 weeks gestational age from women undergoing elective pregnancy termination (n=10 each group). Chorionic plate artery (CPA) segments were obtained from normal term placenta delivered by elective caesarean section (n=13). The study had local ethical approval (National Research Ethics Service Committee Sunderland, ref 07/H0904/74; Newcastle & North Tyneside 1, ref 10/H0906/71) and written informed consent was obtained from all subjects.

Formalin fixed, paraffin embedded placental bed biopsies were sectioned at 3μm and immunostained as described previously to confirm the presence of decidua, myometrium, EVT and SpAs (Robson et al., 2002). Samples selected contained non-remodelled SpAs or SpAs with features of early partial remodelling (dilatation, VSMC separation and morphological change) (Lash et al., 2016).

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections as described previously (Schiessl et al., 2009) using an avidin-biotin peroxidise method (Vectastain Elite, Vector Laboratories, Peterborough, UK). Primary antibody specificities, dilutions, pre-treatments and incubation conditions are shown in Table I. Sections were lightly counterstained with Mayer's haematoxylin (BDH, Poole, UK) and mounted with DPX

synthetic resin (Raymond Lamb, London, UK). Appropriate positive controls were performed in each staining run and a negative control was performed for each sample by replacing the antibody with non-immune serum. For any individual antibody, all the samples to be analysed were stained in a single staining run with the appropriate positive control to avoid day to day variation.

Laser capture microdissection and real time RT-PCR

Laser capture microdissection and RT-PCR was used to confirm the presence or absence of mRNA corresponding to AGF receptor expression in VSMCs identified by immunohistochemistry.

10µm cryosections of placental bed biopsies (n=6) were cut onto 2µm PEN-Membrane slides (Leica Microsystems, Wetzlar, Germany) and stained with cresyl violet LCM staining kit (Ambion, TX, USA) to allow SpA identification. Initially the lumen and endothelial cells were laser microdissected using a Leica AS LMD microscope (Leica Microsystems) and removed as waste. The remaining SpA wall containing VSMCs was then laser microdissected and used for RT-PCR.

Total RNA was extracted using RNAqueous Micro (Ambion) and reverse transcribed to cDNA using Superscript III (InVitrogen, Paisley, UK. Real time RT-PCR was performed using Taqman chemistry, 2µl cDNA per 20µl reaction with Taqman Universal PCR MasterMix (Applied Biosystems, CA, USA) and run on an ABI7000 real time PCR machine (Applied Biosystems). Probe/primer sets were purchased from Assays on Demand (Applied Biosystems) for GAPDH (housekeeping gene), Tie-2, TGF-βRI, TGF-βRII, VEGF-R1, VEGF-R2, VEGF-R3, IFN-γR1 and IFN-γR2. Due to

the very small size of the laser capture dissected samples and the number of genes investigated the concentration of cDNA was extremely limited. PCR reactions were therefore run for 50 cycles. All probe/primer sets were validated for use with a laboratory standard total RNA extracted from early pregnancy decidua.

Isolation and culture of CD56⁺ uNK cells, EVT and uNK cell/EVT co-cultures

Decidua and placenta were taken from the same patient for isolation of both uNK cells and EVTs.

uNK cell-enriched isolates were prepared by enzymatic disaggregation and positive immunomagnetic selection (Miltenyi Biotech, Bisley, UK) as described previously (Lash et al., 2006). To prepare uNK cell-conditioned medium (uNK-CM), cells were cultured at 1x10^6 cells/ml in RPMI1640 complete medium (RPMI containing 10% (v/v) fetal calf serum, ~~1%~~5U/ml penicillin, 0.05mg/ml~~/~~ streptomycin, ~~1%~~1.5µg/ml amphotericin; Sigma-Aldrich). After 24 hours the uNK-CM was removed, centrifuged and stored at -80 ºC. Cell purity and viability were tested routinely by immunostaining cell smears and trypan blue exclusion, respectively; both were consistently >95%.

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EVTs were isolated as previously described (Lash et al., 2010a) and cultured at 1x10^6 cells/ml in DMEM:Ham's F12 (Sigma-Aldrich) complete medium in a 24 well plate coated with growth factor reduced Matrigel (BD Biosciences, Oxford, UK). Conditioned medium was harvested (EVT-CM) after 24 hours, centrifuged and stored at -80 ºC. Cell purity and viability were assessed after 24 hours of culture by immunostaining cell smears (HLA-G, cytokeratin 7) (Lash et al., 2010a) and trypan blue exclusion, respectively; both were consistently >95%.

For co-culture of uNK cells and EVTs, $1x10^6$ EVT cells were cultured in DMEM:F12 complete medium in a 24 well plate overnight prior to addition of $1x10^6$ uNK cells (from the same patient) in DMEM:Ham's F12 to a total volume of 1ml (Lash et al., 2011; Robson et al., 2012). After 24 hours co-culture, the conditioned medium (uNK/EVT-CM) was removed, centrifuged and stored at -80 °C. Viability of co-cultures was assessed after 24 hours of culture and was consistently >95%.

Isolation and culture of chorionic plate arteries

Intact CPA segments (5mm length) (Robson et al., 2012; Pitman et al., 2013; Lash et al., 2016) were dissected from normal term placenta and cultured at 37 °C in 5% CO_2 in: (A) conditioned medium: 20% (v/v) uNK-CM, EVT-CM or uNK/EVT-CM (8-10 or 12-14 weeks' gestation; n=6 each gestational age group and CM type); (B) angiogenic growth factors: 1ng/ml and 10ng/ml VEGF-A, VEGF-C, Ang-1, Ang-2, IFN-γ or TGF-β1 (all from R&D Systems, Abingdon, UK; n=10 each growth factor); (C) conditioned medium and anti-angiogenic growth factor neutralising antibody: 20% (v/v) uNK-CM, EVT-CM or uNK/EVT-CM (8-10 or 12-14 weeks gestation; n=5 each experiment) with or without anti-Ang-1 (1μg/ml; Millipore, Watford, UK), anti-Ang-2 (10μg/ml; Amgen, Seattle, Washington); or (D) medium only controls: RPMI1640 complete medium control and a citric acid control for TGF-β1, which is reconstituted in citric acid. The medium was replaced every 48 hours. CPAs were harvested after 120 hours, fixed in formalin for 24 hours and embedded in paraffin wax. Three μm serial sections were immunostained for: H-cal, MyHC and α-SMA to determine the differentiation state of CPA VSMCs after culture; cleaved caspase-3 to verify cell viability; Ki67 to examine

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cell proliferation in order to ensure that any differences detected were not due to altered cell proliferation; and CD31 to confirm endothelial integrity.

Analysis of immunostaining

For assessment of VSMC differentiation marker expression, digital images of immunostained decidual SpAs and CPAs were captured using a Nikon Eclipse 80i microscope coupled with a DS-Fi1 camera and NIS elements D software. In Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) individual SpAs were selected using the magnetic lasso tool and copied to a new layer within the image, where a positively stained area was identified by eye and selected using the magic wand tool. Other immunopositive areas were then selected using the 'similar' command, with the threshold level set such that all positive areas but no negative areas were selected. The immunopositive areas were then copied into a new layer. The pixel number in each layer was noted and the number of positive pixels in immunostained sections was expressed as a percentage of the total pixel number in the vessel wall for each SpA or CPA segment (Pitman et al., 2013; Lash et al., 2016).

VSMC immunostaining for AGF receptors was assessed in non-remodelled (n=33 SpAs) and partially remodelled (n=33 SpAs) SpAs using a modified quickscore taking into account both percentage of cells stained and intensity of staining (Schiessl et al., 2009). The degree of remodelling was determined in serial sections immunostained for MyHC and cytokeratin 7 with non-remodelled SpA showing no signs of remodelling and partially remodelled SpA displaying separated and rounded VSMCs but no associated EVT.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using the StatView statistical software package (Abacus Concepts, Berkley, CA, USA). Statistical significance was determined using a one-way ANOVA, followed by Fisher's post hoc analysis unless otherwise stated. Differences were considered statistically significant at $P<0.05$.

Results

Uterine spiral arteries express receptors for angiogenic growth factors

We investigated AGF receptor expression by VSMCs in non- and partially remodelled SpAs (Figure 1A). There was moderate to strong expression of VEGF-R1 and VEGF-R2 in SpA VSMCs, with weaker VEGF-R3 expression. TGF-βRI, TGF-βRII and IFN-γR1 were moderately expressed, although IFN-γR2 immunoreactivity was weaker. Tie-2, the receptor for Ang-1 and Ang-2 was weakly to moderately expressed. Scores for most AGF receptors did not differ between non- and partially remodelled SpAs (Figure 1B) but immunostaining was increased in partially remodelled compared with non-remodelled SpAs for VEGF-R2 (P=0.005) and Tie-2 (P=0.0007) (Figure 1A, 1B).

Expression of mRNA for VEGF-R1, VEGF-R2, VEGF-R3, TGF-βRI, TGF-βRII, IFN-γR1, IFN-γR2 and Tie-2 in VSMCs of SpAs was confirmed with laser capture microdissection coupled with real-time RT-PCR in all samples examined (n=6 samples; Figure 1C). PCR reactions were run for 50 cycles and for each gene the cycle number at threshold was higher than ideal. However, all reactions demonstrated classical amplification curves and reached saturation prior to 50 cycles (GAPDH 31, H-cal 35, Tie-2 34, IFNγ-R1 40, IFNγ-R2 34, TGF-βRI 34, TGF-βRII 31, VEGF-R1 26, VEGF-R2 38, VEGF-R3 37).

Vascular smooth muscle cells of partially transformed spiral arteries switch from a contractile to a more synthetic phenotype

Markers of contractile (α-SMA, calponin, H-cal, MyHC, smoothelin) and synthetic (osteopontin) VSMC phenotype were compared between non- and partially remodelled SpAs (n=15 SpAs each group) at 8-14 weeks gestational age (Figure 2A).

Partially remodelled SpAs showed endothelial swelling and separation of medial VSMCs but no intramural or endovascular EVTs, indicating an early stage of SpA remodelling prior to invasion of vessel wall and lumen by EVTs (7); SpAs were classified as non- and partially remodelled by two investigators (JNB, AR).

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Expression of H-cal (P=0.0003), α-SMA (P<0.0001), MyHC (P=0.001), and smoothelin (P=0.0001) was reduced and osteopontin (P=0.008) expression was increased in partially compared with non-remodelled SpAs. In order to correct for the possibility that the number of VSMCs in SpAs was reduced in partially remodelled SpA, immunopositivity for each VSMC marker was also expressed as a percentage of calponin immunopositivity (Figure 2B), an early marker of VSMC differentiation whose expression remained high and showed minimal changes in expression in partially remodelled vessels. When expressed as a percentage of calponin immunoreactivity, expression of α-SMA (P<0.0001), H-cal (P=0.03), MyHC (P=0.03) and smoothelin (P=0.0001) was reduced and osteopontin (P=0.0001) was increased in partially remodelled SpAs compared with non-remodelled SpAs (Figure 2A, 2B). These results suggest that in the early stages of remodelling SpA VSMCs show loss of their contractile phenotype, adopting a more synthetic or dedifferentiated phenotype.

uNK cell, EVT and uNK cell:EVT conditioned medium stimulates VSMC dedifferentiation in chorionic plate arteries in vitro

We tested the hypothesis that uNK cells initiate VSMC dedifferentiation by quantification of α-SMA, H-cal, MyHC and osteopontin immunoreactivity in an *in vitro* CPA model (Robson et al., 2012; Pitman et al., 2013; Lash et al., 2016). Expression

by CPA VSMC of receptors for Ang-1, Ang-2, IFN-γ, TGF-β1, VEGF-A and VEGF-C was initially confirmed by immunohistochemistry (Figure 3C).

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All CPAs were immunostained for caspase 3, Ki67 and CD31 after culture. There was no evidence of caspase 3 immunopositivity, confirming viability. The endothelium was intact as demonstrated by immunostaining for CD31; CD31-negative vessels (reflecting endothelial loss) were excluded from the study. There was minimal Ki67 immunopositivity, confirming that proliferation was not altered after culture. CPAs cultured with uNK-CM, EVT-CM or uNK/EVT-CM from 8-10 weeks gestational age showed no difference in H-cal, MyHC, α-SMA or osteopontin expression (Figure 4A-4D) compared with medium only controls. However, reduced H-cal and MyHC expression was observed after culture in uNK-CM (H-cal $P<0.0001$; MyHC $P=0.03$), EVT-CM (H-cal $P=0.0006$; MyHC $P=0.02$) or uNK/EVT-CM (H-cal $P=0.001$; MyHC $P=0.05$) from 12-14 weeks gestational age (Figure 4A, 4B). Osteopontin expression was increased after culture in uNK-CM ($P=0.03$), EVT-CM ($P=0.01$) and uNK/EVT-CM ($P=0.02$) from 12-14 weeks gestational age (Figure 4D). α-SMA expression was reduced only after culture in uNK-CM ($P=0.02$) from 12-14 weeks gestational age samples (Figure 4C).

Ang-1 and Ang-2 reduce H-cal expression in CPA

To identify whether AGFs produced by uNK cells mediate VSMC dedifferentiation, CPAs were cultured with 1ng/ml and 10ng/ml recombinant AGFs and H-cal expression assessed. There was no effect on H-cal expression after incubation of CPAs with both concentrations of IFN-γ, VEGF-A and VEGF-C (Figure 3A). 1ng/ml Ang-1 and Ang-2 also had no effect but incubation of CPAs in 10ng/ml recombinant human Ang-1

(*P*=0.006) and Ang-2 (*P*=0.009) reduced VSMC expression of H-cal (Figure 3A). TGF-β1 was excluded from this part of the study since the citric acid used to reconstitute TGF-β1 reduced expression of the VSMC marker studied (data not shown).

Neutralisation of uNK cell secreted Ang-1 and Ang-2 activity partially restored contractile VSMC marker expression

Neutralising antibodies to Ang-1 or Ang-2 were added to 12-14 week gestational age uNK-CM and CPAs cultured for 120 hours. Neutralisation of both Ang-1 (*P*=0.0004) and Ang-2 (*P*=0.004) restored H-cal expression (Figure 3B).

Discussion

In the current study we have demonstrated that in early remodelling, in the absence of EVT infiltration, SpA VSMCs show loss of contractile phenotype. Furthermore, AGFs produced by uNK cells and EVTs can mediate this VSMC de-differentiation in an *in vitro* model (Lash et al., 2006, 2010a).

We have demonstrated expression of protein and mRNA for receptors for Ang-1, Ang-2, IFN-γ, TGF-β1, VEGF-A and VEGF-C in uterine SpA VSMCs. Expression of VEGF-R2 and Tie-2 correlate with the high levels of VEGF-C, Ang-1 and Ang-2 secreted by uNK cells in early pregnancy and suggest a role in trophoblast-independent SpA remodelling. Interestingly, VEGF-R2 and Tie-2 expression was increased in partially remodelled SpAs, at a gestational age when levels of uNK cell secreted AGFs are decreasing (Lash et al., 2006). In partially remodelled SpAs there is separation and some dedifferentiation of VSMCs, but they are not yet lost. Increased receptor expression may reflect altered VSMC phenotype as they prepare to migrate away from the SpA. Despite high VEGF-R1 expression in SpA VSMCs, uNK secretion of VEGF-A is low, although EVTs secrete high levels of this AGF (Lash et al., 2010a). Taken together, these data confirm that SpAs express AGF receptors and are a potential target for uNK cell derived factors.

The main function of VSMCs is to control vessel contraction, thereby regulating blood vessel tone and blood pressure. VSMCs exhibit plasticity and can dedifferentiate in response to local environmental signals during vascular development or vascular injury (Owens, 1995, 2007). VSMCs acquire specific markers as they differentiate to a contractile phenotype, which are lost when they dedifferentiate into a more synthetic

phenotype. Regulation of VSMC differentiation/dedifferentiation is complex and involves local cues including mechanical forces, extracellular matrix interactions, local cytokines, growth factors and epigenetic control (Owens et al., 2004; Alexander and Owens, 2012). α-SMA is the first contractile marker expressed during development, at approximately embryonic day 2.5 in chick (Owens, 1995), and is the most abundant actin isoform in mature differentiated VSMCs (Fatigati and Murphy, 1984). H-cal is the most abundantly expressed caldesmon isoform in differentiated VSMC and is a later marker of VSMC differentiation than α-SMA (Sobue and Sellers, 1991; Stouffer and Owens, 1994), being expressed at around embryonic day 6 in chick (Owens, 1995). MyHC is part of the contractile apparatus in all muscle cells and consists of two isoforms SM-1 and SM-2 produced by alternative splicing. These isoforms are late markers of VSMC differentiation, with SM-2 expressed ~10 days postpartum (Fatigati and Murphy, 1984; Kuro-o et al., 1989). Calponin is a regulator of VSMC contraction and is a relatively early marker of VSMC differentiation expressed from day 4 in chick embryo (Owens, 1995). Smoothelin is a late VSMC differentiation marker and mouse knockout studies suggest that it is required for contractile potential (van Eys et al., 2007). Osteopontin is a secreted acidic glycosylated phosphoprotein that interacts with integrins and extracellular matrix; expression is increased in dedifferentiated VSMCs and osteopontin regulates expression of α-SMA and calponin (Gao et al., 2012).

We demonstrated reduced immunostaining for α-SMA, H-cal, MyHC and smoothelin within the walls of SpAs undergoing early remodelling prior to EVT infiltration. These data suggest that in early remodelling SpA VSMCs become less contractile; this reduced expression of contractile proteins is similar to that reported in vascular

diseases such as atherosclerosis (Ross, 1993) and may account for the dilatation in early SpA remodelling (Craven et al., 1998; Pijnenborg et al., 2006). Osteopontin expression, indicating synthetic VSMC phenotype (Weissgerber et al., 2010), was increased in the same SpAs. The functional importance of this increased osteopontin expression is unknown but osteopontin promotes VSMC migration *in vitro* (Li et al., 2007). We have demonstrated that VSMCs migrate away from the SpA wall during early SpA transformation (Bulmer et al., 2012); this may reflect a synthetic phenotype capable of migration. This is the first demonstration of phenotypic changes in SpA VSMCs in early SpA remodelling in the absence of EVT and supports evidence from guinea pig pregnancy of dedifferentiation of uterine artery VSMCs (Nanaev et al., 1995). A recent study has reported that predecidual stromal cells express pericyte markers, including α-SMA and the contribution of these cells to decidual SpA remodelling should also be considered (Munoz-Fernandez et al., 2018).

Using a CPA model we assessed the effects of uNK cell, EVT and uNK cell/EVT conditioned medium on VSMC dedifferentiation *in vitro*. Both uNK cells and EVT supernatants reduced VSMC expression of H-cal, MyHC and α-SMA and increased osteopontin expression. Although MyHC is the latest VSMC differentiation marker of the three examined, H-cal showed most loss in expression. A possible explanation is that the MyHC antibody recognises both SM-1 and SM-2; these isoforms may show differential regulation, although this cannot be concluded from our data. In a study using an aorta VSMC cell line, α-SMA and calponin expression were decreased after culture in EVT-CM (Wallace et al., 2013). The authors hypothesised that this may be attributed to CXCL10, although this chemokine stimulated VSMC motility but had no effect on VSMC contractile protein expression (Wallace et al., 2013).

Interestingly we only observed contractile muscle protein loss after culture with supernatants from 12-14 weeks' but not 8-10 weeks' gestation. Both groups secrete high levels of AGFs, although levels of Ang-2 and VEGF-C were reduced at 12-14 weeks' compared with 8-10 weeks' gestation (Lash et al., 2006). A possible explanation is that the SpA VSMCs must first undergo separation of the previously intact layers; this process is initiated by 8-10 weeks' gestation uNK cell culture supernatants (Robson et al., 2012). We also observed that EVT-CM and uNK cell/EVT-CM reduced expression of H-cal and MyHC, but not α-SMA, although for H-cal the effect of EVT-CM and uNK cell/EVT-CM was not as potent as that of uNK-CM. These data suggest that close proximity of uNK cells and EVT cells may lead to mutual suppression of function; previous data from our laboratory demonstrated reduced secretion of several cytokines and AGFs in uNK cell/EVT co-cultures compared with cultures of individual cell types (Lash et al., 2011). The gestational age differences in the effect of the uNK cell and EVT supernatants suggests that this is not a non-specific effect. Furthermore, isolated decidual macrophages did not affect VSMC dedifferentiation in the CPA model, despite their production of high levels of AGFs (Lash et al., 2016).

Ang-1 and Ang-2 reduced expression of H-cal in the CPA model and addition of Ang-1 and Ang-2 neutralising antibody abrogated the effect of uNK-CM on H-cal expression. This is one of the first descriptions of Ang-1 and Ang-2 playing similar roles and having biological effects on VSMCs. The mechanism of action warrants further study.

We propose a working model of the cells and molecular mediators involved in SpA remodelling. In early gestation SpAs are surrounded by uNK cells and macrophages which facilitate breakdown of extracellular matrix in the vessel wall via matrix metalloproteinase secretion (Hazan et al., 2010; Robson et al., 2012; Lash et al., 2016). Matrix metalloproteinases have also been localised within the SpA wall itself (Hazan et al 2010). uNK cells may then induce VSMC rounding up and separation via AGFs including Ang-2 (Robson et al., 2012; Pitman et al., 2013). uNK cells also attract EVTs towards the SpAs via secretion of IL-8, CXCL10 and other cytokines (Hanna et al., 2006; Lash et al., 2010b). EVT and uNK cells interact via EVT expression of HLA-C, potentially influencing their function (Xiong et al., 2013). Both uNK cells and EVT may then induce dedifferentiation of VSMCs, through AGF secretion, particularly Ang-1 and Ang-2. This synthetic phenotype increases their motility and VSMC migrate into the stroma surrounding remodelling SpAs where they undergo apoptosis and are phagocytosed by resident macrophages (Bulmer et al., 2012; Lash et al., 2016). Disruptions in any of these cell types and molecular mediators may contribute to failed SpA remodelling and hence to the pathogenesis of pregnancy complications.

Acknowledgements

The authors thank Amgen for the Ang-2 neutralising antibody and staff on the delivery suite at the Royal Victoria Infirmary for help consenting and collecting samples. The authors also thank all those women who donated samples for these studies.

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Author Roles

AR performed experiments, analysed data and wrote the manuscript; GEL conceived the study, performed experiments and edited the manuscript; BAI performed experiments; SCR collected samples; JNB conceived the study and edited the manuscript.

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Conflict of interest

None

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Table I: Primary antibodies for immunohistochemistry

620

Antibody	Pre-Treatment	Dilution	Incubation Time	Species	Company	Clone/ Cat#
Tie-2	EDTA[1]	1:200	60 mins	Goat	R&D, Abingdon, UK	AF313
IFN-γR2	EDTA	1:100	60 mins	Mouse	Abcam, Cambridge, UK	31606
IFN-γR1	Nil	1:50	60 mins	Rabbit	Abcam	61179
VEGF-R1	Citrate[2]	1:200	60 mins	Rabbit	Insight Biotech, Wembley, UK	9029
VEGF-R2	Citrate	1:100	60 mins	Mouse	Insight Biotech	6251
VEGF-R3	Citrate	1:500	60 mins	Rabbit	Insight Biotech	321
TGF-βRI	EDTA	1:50	60 mins	Rabbit	Abcam	31013
TGF-βRII	Citrate	1:100	30 mins	Rabbit	Abcam	28382
H-caldesmon	Citrate	1:100	60 mins	Mouse	Dako, Ely, UK	M3557
Calponin	Trypsin[3]	1:80	30 mins	Mouse	Dako	M3556
Myosin Heavy Chain	Citrate	1:600	60 mins	Mouse	Sigma-Aldrich, Poole, UK	M7786
α-smooth muscle actin	Trypsin	1:75	30 mins	Mouse	Leica, Milton Keynes, UK	SMA
Smoothelin	Citrate	1:100	60 mins	Mouse	Abcam	8969
Cleaved-caspase 3	Citrate	1:200	30 mins	Mouse	Cell Signalling, New England Biolabs, Hitchin, UK	9661
Ki67	Citrate	1:200	30 mins	Mouse	Leica	MMI
CD31	Citrate	1:20	60 mins	Mouse	Leica	IAIO

[1]EDTA: pressure cook for 1 min in EDTA buffer pH 8
[2]citrate: pressure cook for 1 min in citrate buffer pH 6
[3]trypsin: incubate in 0.1% trypsin for 10 mins at 37˚C

Figure Legends

Figure 1: Angiogenic growth factor receptors during spiral artery remodeling **A)** Representative photomicrographs of immunostaining for angiogenic growth receptors in non-remodelled (top 3 rows) and partially remodelled (bottom row) superficial myometrial spiral arteries in placental bed biopsies. The antibodies used are shown on the photomicrographs. Immunoreactivity for vascular endothelial growth factor receptor 2 (VEGF-R2) and Tie-2 is increased in the wall of the partially remodelled artery compared with the non-remodelled artery. Scale bar 50μM. **B)** Graphical representation of quickscore for receptor expression in non-remodelled and partially remodelled spiral arteries. **C)** Non-transformed spiral arteries were laser capture microdissected from sections of placental bed biopsies stained with cresyl violet. Real time RT-PCR was performed for GAPDH, H-caldesmon (Hcal), VEGF-R1, VEGF-R2, VEGF-R3, transforming growth factor beta receptors (TGFβ-R1, TGFβ-R2), interferon gamma receptors (IFNγ-R1, IFNγ-R2) and Tie-2. As there is no comparator, data are shown as delta Ct relative to GAPDH. Note that the lower the number the more abundant the mRNA. Data are shown as mean±SEM.

Figure 2: Loss of contractile phenotype during spiral artery remodelling **A)** Representative photomicrographs of ~~immunostaining for cytokeratin 7 (demonstrating absence of trophoblast)~~, α-SMA, H-Cal, MyHC, osteopontin and~~,~~ calponin ~~and smoothelin~~ in vascular smooth muscle cells of non-remodelled and partially remodelled spiral arteries in placental bed biopsies. Scale bar 50μM. **B)** Quantification of α-SMA, H-Cal, MyHC, osteopontin and smoothelin in vascular smooth muscle cells of non-remodelled (n=15) and partially remodelled (n=15) spiral arteries normalised to calponin expression. Statistical significance was determined by use of an unpaired t-Test.

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CHECKED AND FINE

Commented [CF9]: Please confirm that this is correct. We need to know which images refer to what stage of SpA development. Otherwise add labels like Figure 2.
APOLOGIES – YES THIS IS CORRECT AND I HAVE ADDED (TOP 3 ROWS) FOR THE NON-REMODELLED ARTERIES

Commented [CF10]: Nowadays, many authors do not define abbreviations for protein names. However, you have done so elsewhere in the paper and I think it is good practice. Human Reproduction tries to make figures with their legend intelligible without refence elsewhere so logically abbreviations have to be defined even if already defined elsewhere.
THANKS.

Commented [JB11]: I am not sure why this has been deleted. The cytokeratin 7 staining was added at the request of one of the reviewers.

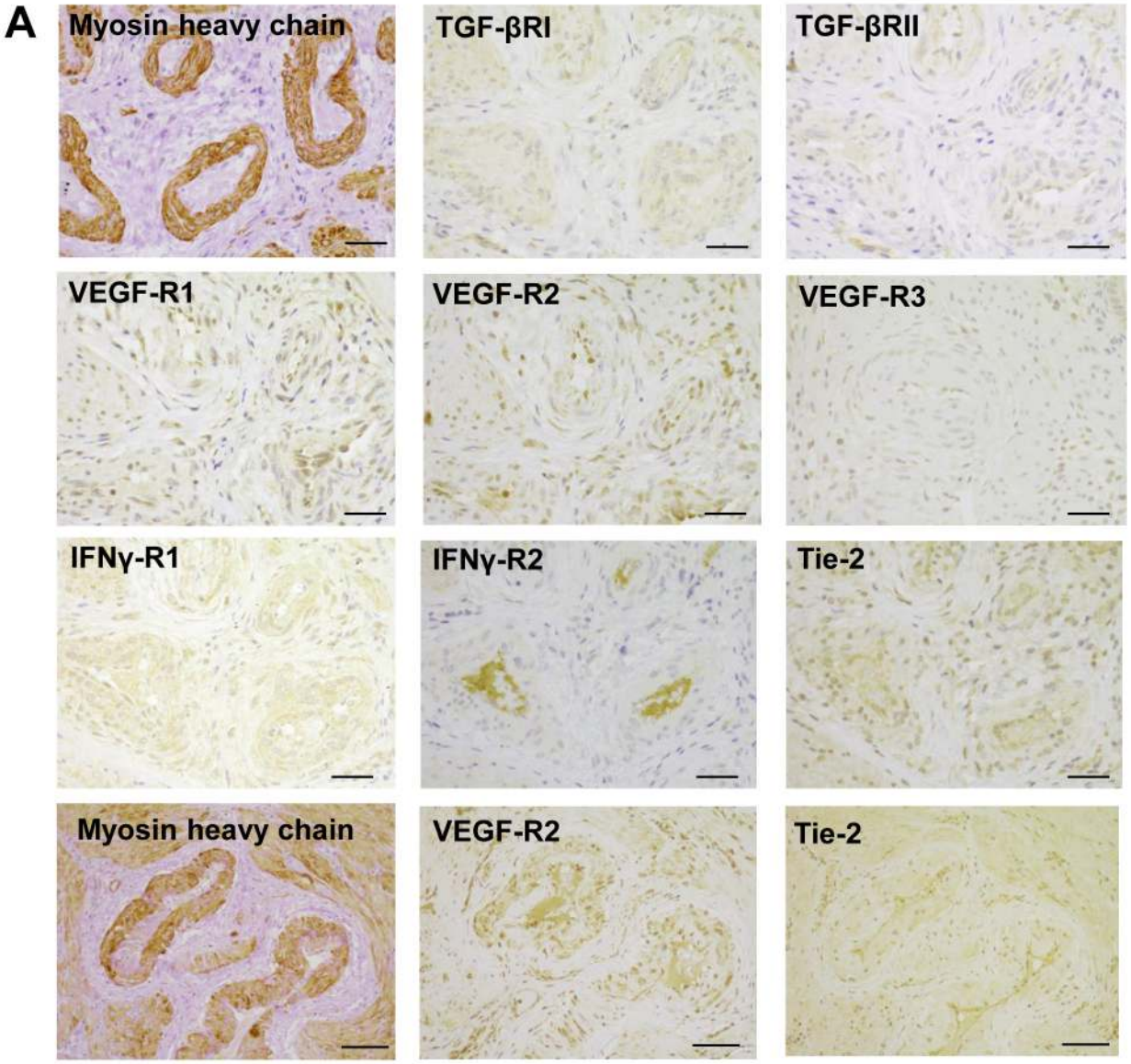
Commented [JB12]: We omitted the smoothelin immunostain to add the cytokeratin 7 – apologies for the error

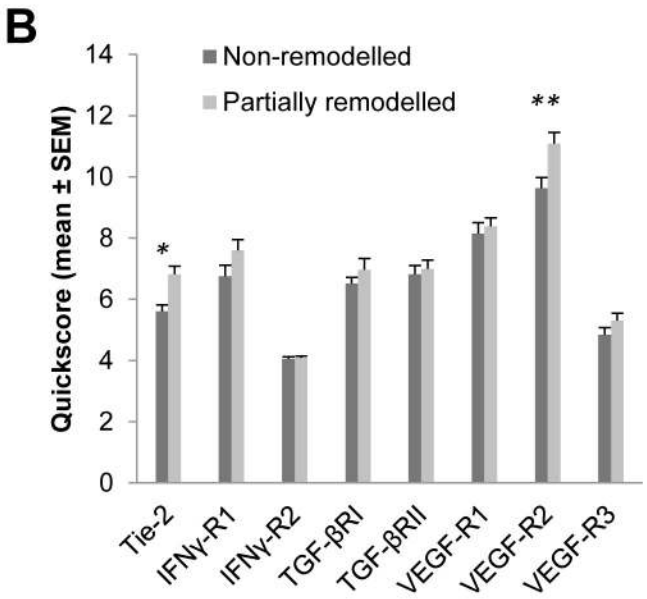
Figure 1

Figure 3: Vascular smooth muscle cell dedifferentiation by uterine natural killer cell derived growth factors **A)** Chorionic plate arteries (CPAs) were incubated with recombinant human growth factors (0, 1 or 10ng/ml) for 120 hours. Transverse sections of each artery were stained with H-Cal and quantified for amount of expression using Adobe Photoshop. Statistical significance was determined by use of an ANOVA with a Fisher's post-hoc test, n=8 each group. **B)** CPAs were incubated in uterine natural killer cell conditioned medium (uNK-CM) in the presence or absence of Ang-1 or Ang-2 neutralising antibodies (Ab; 1-2µg/ml) for 120 hours. Transverse sections of each artery were stained with H-Cal and quantified for amount of expression using Adobe Photoshop. Statistical significance was determined by use of an ANOVA with a Fisher's post-hoc test, n=5 each group. **C)** Immunostaining of CPAs for Tie-1 and Tie-2. Note immunopositivity of VSMCs.

Figure 4: Dedifferentiation of chorionic plate arteries (CPAs). CPAs were incubated with unconditioned medium (control; 20% (v/v)), uterine natural killer cell conditioned medium (uNK-CM), extravillous trophoblast (EVT)-CM or uNK/EVT-CM for 120 hours from either 8-10 or 12-14 weeks gestational age. Transverse sections of each artery were stained with H-Cal **(A)**, MyHC **(B),** α-SMA **(C)** or osteopontin **(D)** and quantified for amount of expression using Adobe Photoshop. Statistical significance was determined by use of an ANOVA with a Fisher's post-hoc test, n=6 each group.

Figure 1


A
Myosin heavy chain
TGF-βRI
TGF-βRII
VEGF-R1
VEGF-R2
VEGF-R3
IFNγ-R1
IFNγ-R2
Tie-2
Myosin heavy chain
VEGF-R2
Tie-2


B
Non-remodelled
Partially remodelled
Quickscore (mean ± SEM)
0
2
4
6
8
10
12
14
*
**
Tie-2
IFNγ-R1
IFNγ-R2
TGF-βRI
TGF-βRII
VEGF-R1
VEGF-R2
VEGF-R3

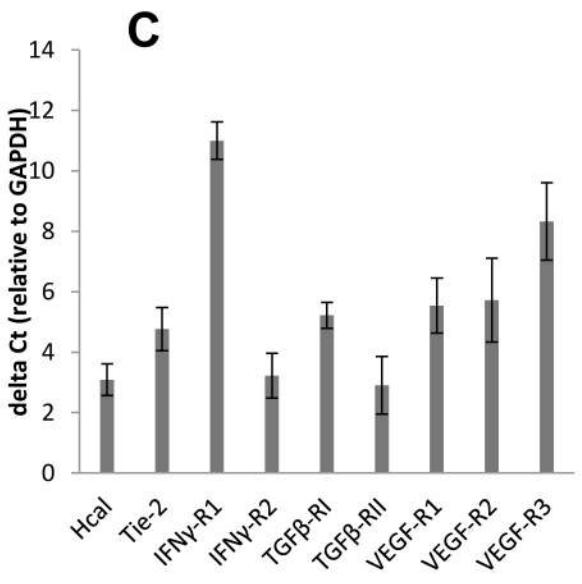
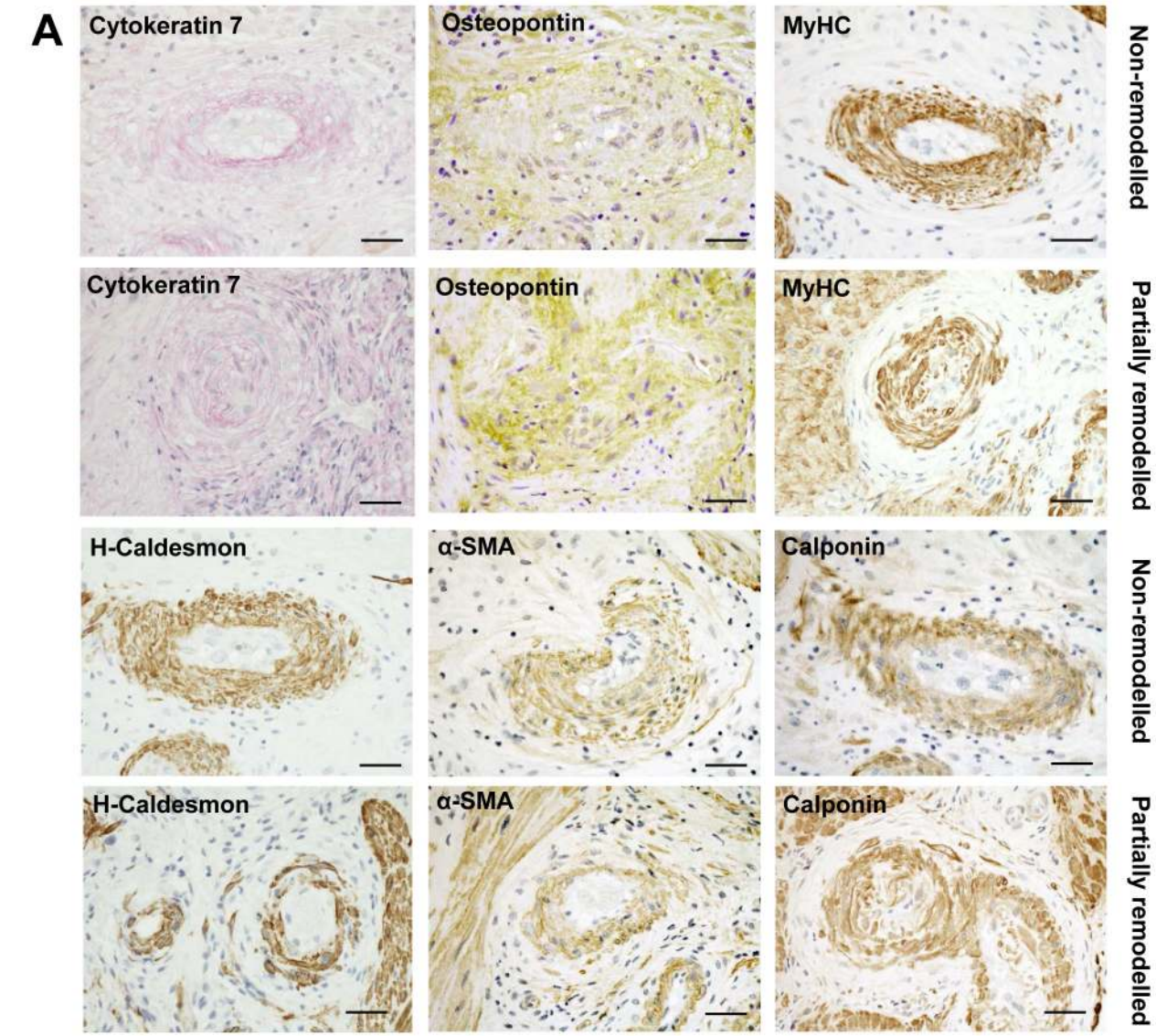

C
delta Ct (relative to GAPDH)
0
2
4
6
8
10
12
14
Hcal
Tie-2
IFNγ-R1
IFNγ-R2
TGFβ-RI
TGFβ-RII
VEGF-R1
VEGF-R2
VEGF-R3

Figure 2


A
Cytokeratin 7
Osteopontin
MyHC
Non-remodelled
Cytokeratin 7
Osteopontin
MyHC
Partially remodelled
H-Caldesmon
α-SMA
Calponin
Non-remodelled
H-Caldesmon
α-SMA
Calponin
Partially remodelled

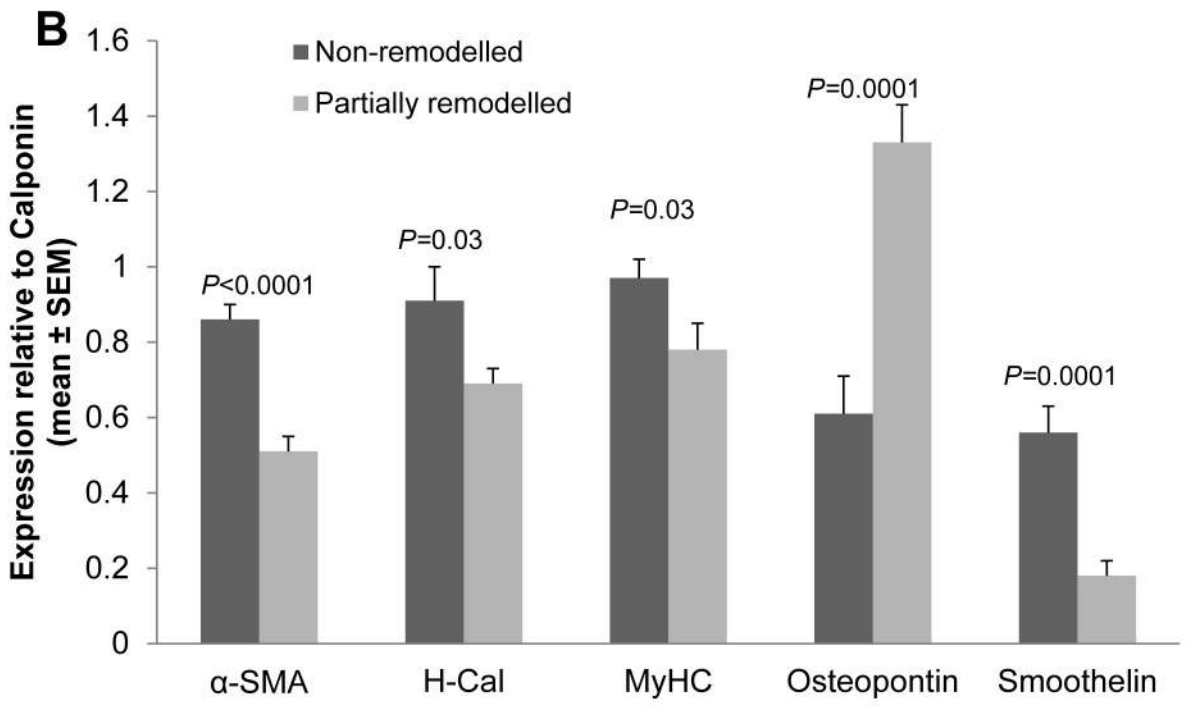
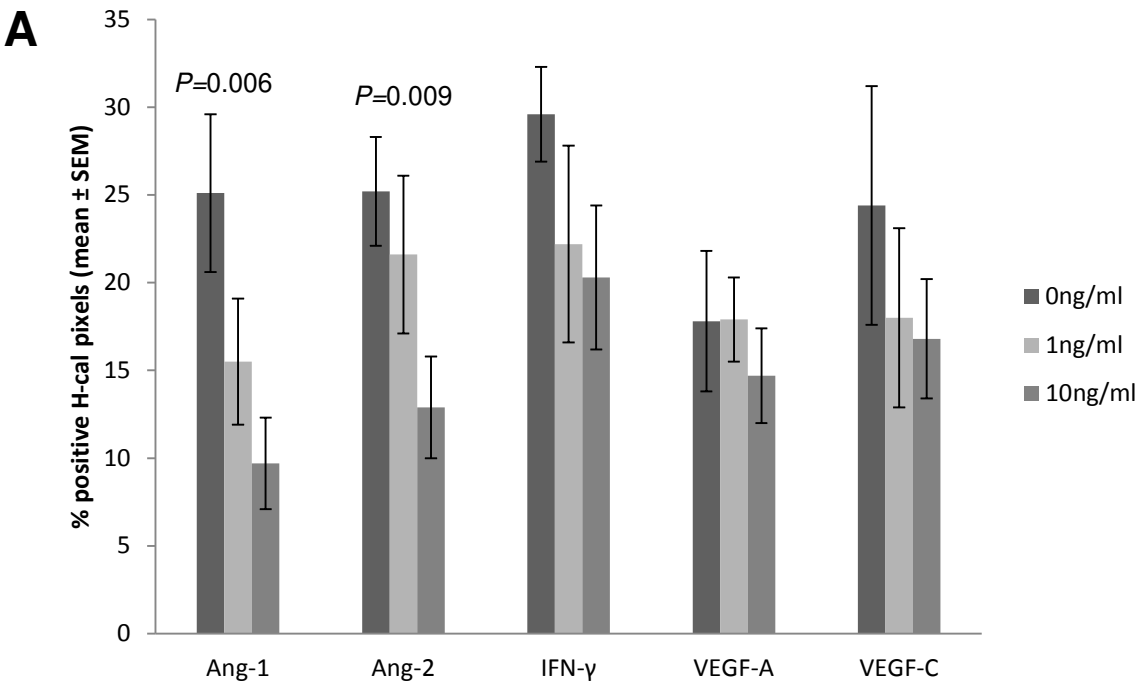
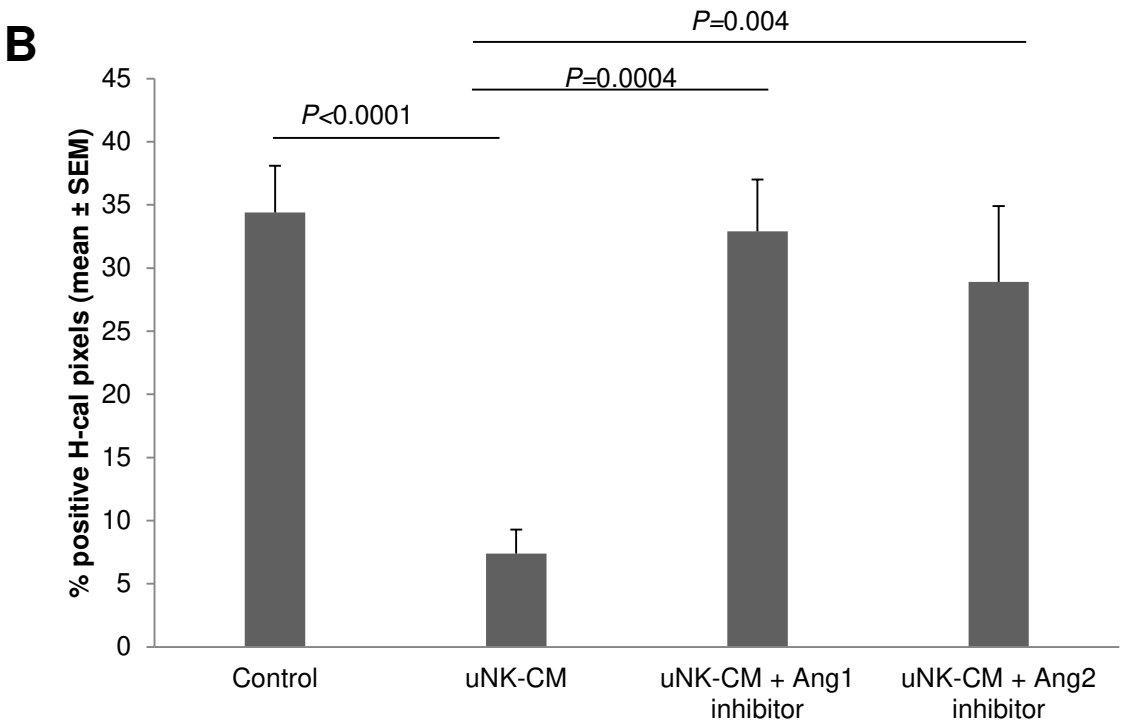

B
Non-remodelled
Partially remodelled
Expression relative to Calponin (mean ± SEM)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P<0.0001
P=0.03
P=0.03
P=0.0001
P=0.0001
α-SMA
H-Cal
MyHC
Osteopontin
Smoothelin

Figure 3


A
35
30
25
20
15
10
5
0
% positive H-cal pixels (mean ± SEM)
P=0.006
P=0.009
0ng/ml
1ng/ml
10ng/ml
Ang-1
Ang-2
IFN-γ
VEGF-A
VEGF-C


B
45
40
35
30
25
20
15
10
5
0
% positive H-cal pixels (mean ± SEM)
P=0.004
P=0.0004
P<0.0001
Control
uNK-CM
uNK-CM + Ang1 inhibitor
uNK-CM + Ang2 inhibitor

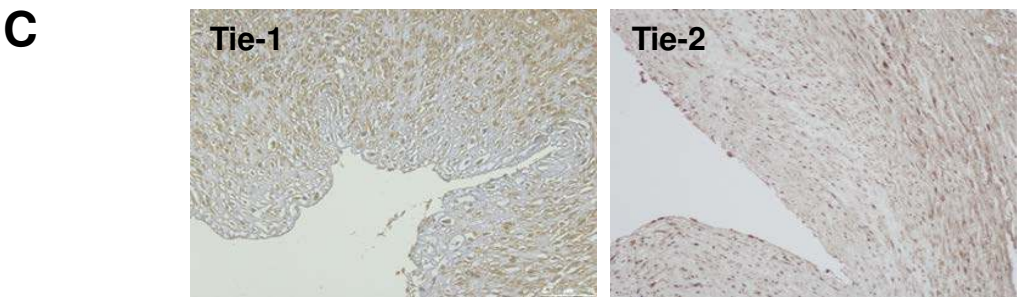
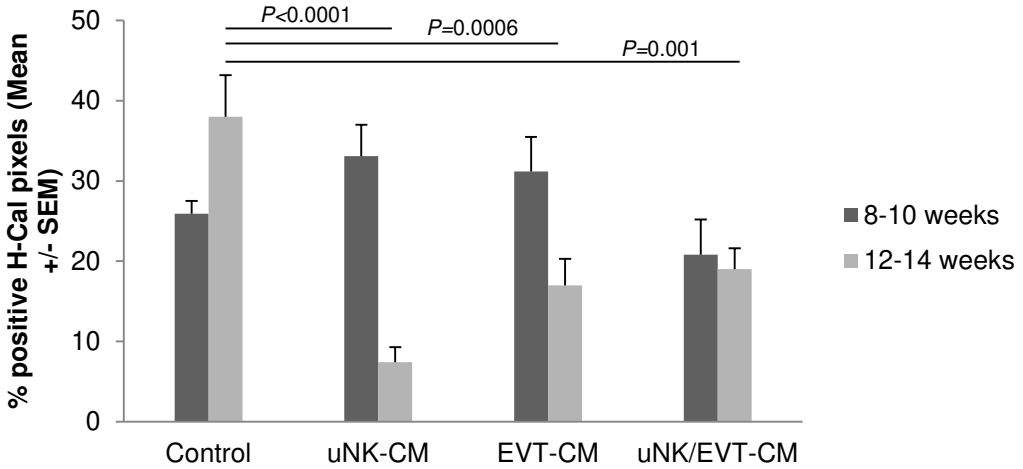
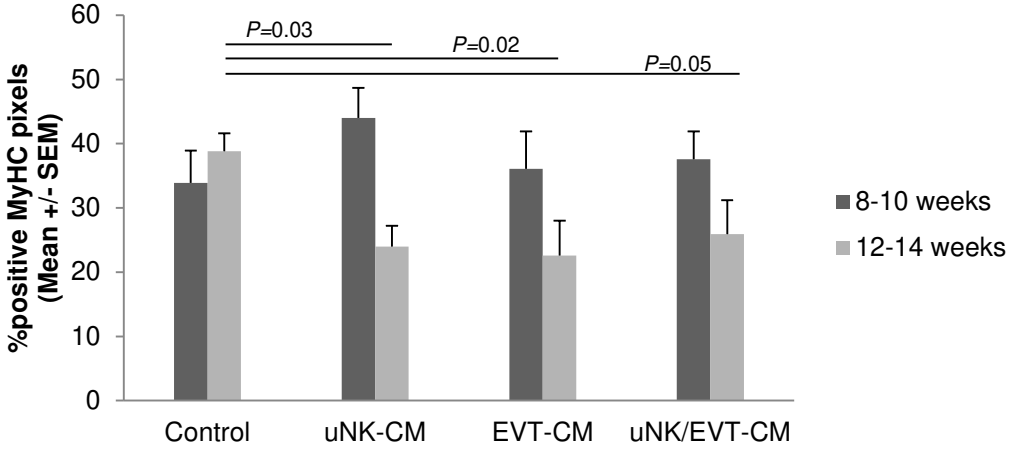

C
Tie-1
Tie-2

Figure 4

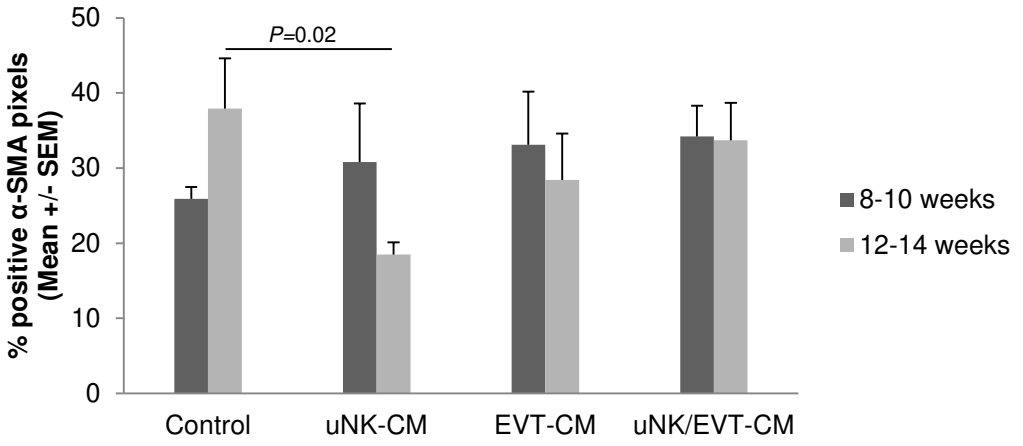
A

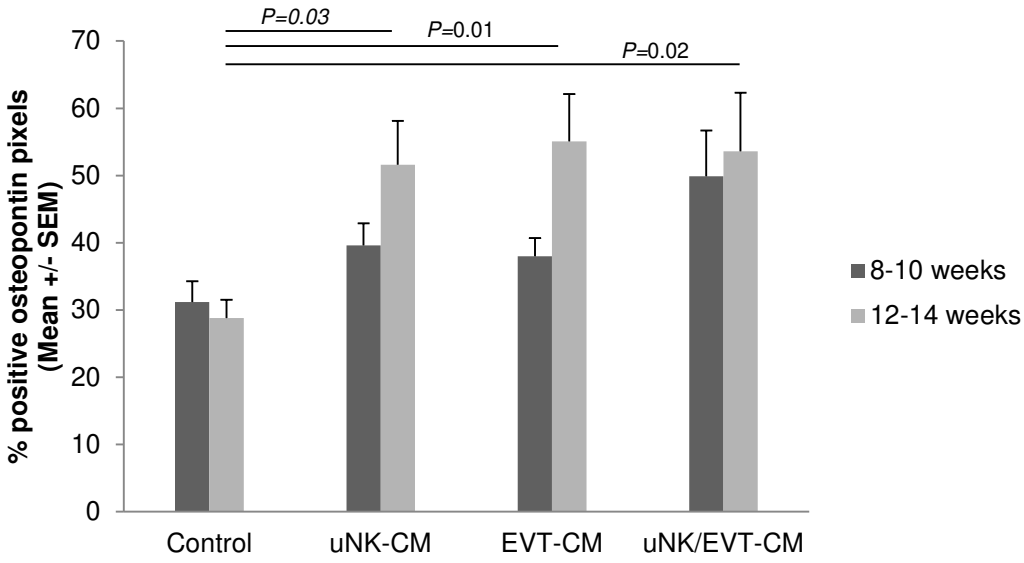

P<0.0001
P=0.0006
P=0.001
% positive H-Cal pixels (Mean +/- SEM)
50
40
30
20
10
0
Control
uNK-CM
EVT-CM
uNK/EVT-CM
8-10 weeks
12-14 weeks

B


P=0.03
P=0.02
P=0.05
%positive MyHC pixels (Mean +/- SEM)
60
50
40
30
20
10
0
Control
uNK-CM
EVT-CM
uNK/EVT-CM
8-10 weeks
12-14 weeks

C


P=0.02
% positive α-SMA pixels (Mean +/- SEM)
50
40
30
20
10
0
Control
uNK-CM
EVT-CM
uNK/EVT-CM
8-10 weeks
12-14 weeks


P=0.03
P=0.01
P=0.02
% positive osteopontin pixels (Mean +/- SEM)
70
60
50
40
30
20
10
0
Control
uNK-CM
EVT-CM
uNK/EVT-CM
8-10 weeks
12-14 weeks