

## ORIGINAL RESEARCH ARTICLE

## Nociceptin/Orphanin FQ receptor expression in primary human umbilical vein endothelial cells is not regulated by exposure to breast cancer cell media or angiogenic stimuli

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### Abstract

**Background:** Opioid receptors are naloxone-sensitive (MOP [ $\mu$ :  $\mu$ ], DOP [ $\delta$ :  $\delta$ ], and KOP [ $\kappa$ :  $\kappa$ ]) and naloxone-insensitive Nociceptin/Orphanin FQ (N/OFQ) peptide receptor (NOP). Clinically, most opioid analgesics target MOP. Angiogenesis is the formation of new blood vessels and involves endothelial cell activation, proliferation, and migration. The effect of opioids on this process is controversial with no data for NOP receptor ligands.

**Methods:** We used patient-derived human umbilical vein endothelial cells (HUVECs) treated with media from the Michigan Cancer Foundation-7 (MCF-7) breast cancer cells or vascular endothelial growth factor (VEGF; 10 ng ml<sup>-1</sup>) and fibroblast growth factor (FGF; 10 ng ml<sup>-1</sup>) as angiogenic stimuli. We have measured (i) NOP/MOP messenger RNA, (ii) receptor protein using N/OFQ<sub>ATTO594</sub> and Dermorphin<sub>ATTO488</sub> as fluorescent probes for NOP and MOP, and (iii) NOP/MOP function in a wound healing assay (crude measure of migration that occurs during angiogenesis).

**Results:** HUVEC lines from 32 patients were used. Using all 32 lines, mRNA for NOP but not MOP was detected. This was unaffected by media from MCF-7 cells or VEGF/FGF. There was no binding of either N/OFQ<sub>ATTO594</sub>(NOP) or Dermorphin<sub>ATTO488</sub>(MOP) in the absence or presence of angiogenic stimuli (six lines tested). In the absence of MOP mRNA, this was expected. Whilst MCF-7 conditioned medium (not VEGF/FGF) reduced wound healing *per se* (14 lines tested), there was no effect of N/OFQ (NOP ligand) or morphine (MOP ligand).

**Conclusions:** Media from MCF-7 breast cancer cells or VEGF/FGF as angiogenic stimuli did not influence NOP translation into receptor protein. MOP was absent. In the absence of constitutive or inducible MOP/NOP, there was no effect on wound healing as a measure of angiogenesis.

**Keywords:** angiogenesis; cancer; confocal microscopy; HUVE cells; nociceptin/orphanin FQ receptors (NOP)

Opioid receptors belong to the family of G protein-coupled receptors. They are subdivided into the classical naloxone-sensitive MOP ( $\mu$ :  $\mu$ ), DOP ( $\delta$ :  $\delta$ ), and KOP ( $\kappa$ :  $\kappa$ ) and the non-classical naloxone-insensitive Nociceptin/Orphanin FQ (N/OFQ) peptide (NOP) receptor.<sup>1,2</sup> Opioid drugs are commonly used in cancer surgery and in palliative care of

patients with cancer who have moderate to severe pain. However, the effects of these drugs on cancer progression are controversial. Opioid receptors are expressed in the tumour microenvironment, and it has been suggested that angiogenesis, invasion, and growth are regulated by opioid receptor activation.<sup>3,4</sup> As a consequence, opioid drugs may enhance

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cancer spread; this is of anaesthetic relevance. Evidence is unclear, and protective effects of opioid drugs have also been described<sup>5,6</sup> most recently in colon adenocarcinoma.<sup>7</sup>

Despite the fact that all opioids produce analgesia to varying degrees, clinical focus is on the MOP receptor. DOP and KOP agonists have been described to support anti-nociception in animal models; however, there is little clinical development.<sup>8,9</sup> The NOP receptor is gathering attention<sup>1,2</sup> for which the agonist cebranopadol is in late-phase development.<sup>10</sup> Data from animal studies suggest that activation of NOP receptors is analgesic without producing tolerance or significant respiratory effects.<sup>11</sup> This system, therefore, offers some clinical advantages.

Angiogenesis is the physiological process through which new blood vessels form and involves endothelial cell activation, proliferation, and migration, and it is a vital mechanism in growth and development, in wound healing, and in the formation of granulation tissue.<sup>12</sup> Apart from its beneficial physiological effects, angiogenesis is essential for development of malignancy. By releasing various growth factors and proteins, tumours induce blood vessel formation. Growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), can induce capillary growth into the tumour providing substrates for tumour expansion.<sup>13,14</sup>

Opioid effects on angiogenesis are controversial.<sup>6,14</sup> Some data suggest that VEGF secreted from tumours increases the expression of MOP in endothelial cells. Morphine stimulates the VEGF receptor and its associated downstream signalling pathways, leading to cell proliferation and vessel formation. A VEGF receptor inhibitor blocked the effects of VEGF, whereas naloxone, an opioid antagonist, did not.<sup>14</sup> There are also studies suggesting inhibition of tumour angiogenesis by opioids. Chronic subcutaneous application of morphine in mouse models caused inhibition of tumour vascularisation through a MOP-dependent HIF1 $\alpha$ /p38 mitogen activated protein kinase (MAPK) pathway.<sup>15</sup> Another mechanism of this inhibition was through leucocyte transmigration. Chronic exposure to morphine could block the regulation of leucocyte transmigration by MOPs.<sup>16</sup>

The effects of MOP receptors in cancer vary between species, tumour types, and drugs used. Importantly, there are no data for NOP receptors, and this would be of interest for the development of new NOP/MOP drugs, such as cebranopadol. In the present study, we have used patient-derived human umbilical vein endothelial cells (HUVECs) and examined (i) the expression of NOP and MOP mRNA by polymerase chain reaction (PCR), (ii) NOP and MOP receptor protein expression using two fluorescent probes, and (iii) NOP and MOP function in a wound healing (a crude measure of migration in angiogenesis) assay. We have treated cells with media from the Michigan Cancer Foundation-7 (MCF-7) breast cancer cells or VEGF/FGF as prototypical angiogenic stimuli.

## Methods

### Cell culture

Umbilical cords were provided by the Anthony Nolan Trust, who obtained ethical approval (East Midlands-Derby; UK Research Ethics Committee; 20/EM/0028) and written informed consent from the donors' parents. The parents also specifically consented to research use of the cord blood/tissue separately. As a condition of our approval, harvested material is anonymised, and no clinical data are available. HUVECs were isolated from the umbilical vein, as described by Jaffe and colleagues<sup>17</sup> with further detail in Bird and colleagues.<sup>18</sup> In total, we used

cells from 32 donors; each donor produced one cell line. These were treated as unique and not combined. HUVECs were cultured in gelatine 0.2%-coated T75 flasks, Thermo-Fisher, UK in media 199 supplemented with fetal calf serum (FCS) 10%, penicillin 100 IU ml<sup>-1</sup>, streptomycin 100  $\mu$ g ml<sup>-1</sup>, fungizone 2.5  $\mu$ g ml<sup>-1</sup>, endothelial cell growth supplement 4.5  $\mu$ g ml<sup>-1</sup>, FGF-acidic 5 ng ml<sup>-1</sup>, heparin 10 U ml<sup>-1</sup>, and thymidine 2.5  $\mu$ g ml<sup>-1</sup>.<sup>18</sup> Cells were cultured at 37°C in CO<sub>2</sub> 5%/humidified air and processed at passages 4–6. As positive controls for NOP and MOP expression, we have used human embryonic kidney (HEK) cells expressing high levels of recombinant human NOP and MOP receptors; these were cultured, as described by Bird and colleagues.<sup>19</sup>

### MCF-7 conditioned media

MCF-7 breast cancer cells were cultured in minimal essential medium supplemented with FCS 10%, penicillin 100 IU ml<sup>-1</sup>, streptomycin 100  $\mu$ g ml<sup>-1</sup>, and fungizone 2.5  $\mu$ g ml<sup>-1</sup>. At 60% confluence, cells were washed once with phosphate-buffered saline (PBS) and serum starved for 24 h. Media were then collected after 24 h and centrifuged at high speed for 10 min, and 5 ml aliquots were stored at –80°C if not used immediately.

### RNA extraction and RT–qPCR to measure NOP and MOP mRNA

HUVECs were used at 80% confluence. Cells were washed once with PBS and treated with VEGF (10 ng ml<sup>-1</sup>)<sup>20</sup> and FGF (10 ng ml<sup>-1</sup>)<sup>20</sup> for either 24 or 48 h, or 50% and 75% conditioned media from MCF-7 breast cancer cells with FCS 1% for 24 h. Cells were incubated at 37°C. Samples from untreated cells were also included. After incubation, cells were washed once with PBS, trypsinised, and centrifuged for 4 min at 400  $\times$  *g*, and TRI Reagent® 1 ml (T9424; Sigma-Aldrich, Gillingham, UK) was added to each cell pellet. Samples were stored in –80°C if not processed immediately. Total RNA was extracted using a phenol–chloroform method.<sup>21</sup> Chloroform 200  $\mu$ l was added to each sample and vortexed intermittently for 2 min until a milky solution was formed. After a further 3-min incubation, the samples were centrifuged for 15 min, the upper aqueous phase was transferred to a clean tube, 2-propanol 500  $\mu$ l was added, and the samples were vortexed. After a 10-min incubation at room temperature, the samples were centrifuged for 10 min, supernatant was removed, and the RNA pellet was resuspended in ethanol 70%. After a brief vortex, the samples were centrifuged for 5 min, and the supernatant was removed. The pellet was allowed to air dry for 5 min and then resuspended in PCR-grade water. NanoDrop™ 2000 (Thermo-Fisher, UK) was used to determine RNA concentration with purity assessed from 260/280 nm ratio (>1.8). All centrifugations were performed at full speed using a cold centrifuge (4°C).

Isolated RNA was processed using a TURBO DNA-free™ kit (Thermo Fisher Scientific) as per manufacturer's instructions to remove genomic DNA contamination. Reverse transcription then followed using the high-capacity reverse transcription kit (Thermo Fisher Scientific). Quantitative PCR was used to assess mRNA quantity using TaqMan and primer limited MGB probes (Thermo Fisher Scientific).<sup>18</sup> We used ELF2B1 (Hs00426752\_m1) and B2M (Hs99999907\_m1) as reference genes, and MOP (Hs01053957\_m1) and NOP (Hs00173471\_m1) receptor probes for our genes of interest. A qPCR thermal profile of 2 min at 50°C, 10 min at 95°C, 50 cycles of 15 s at 95°C,

and 1 min at 60°C was used in a StepOne™ instrument (Applied Biosystems, Warrington, UK).

### Live-cell imaging to measure NOP and MOP receptor expression

In all experiments, coverslips (28 mm Menzel™ glaser #1 coverslips; Thermo Fisher Scientific) were sterilised in ethanol and coated with gelatine 0.2%. Coverslip cultures of HUVECs were treated with VEGF 10 ng ml<sup>-1</sup> and FGF 10 ng ml<sup>-1</sup>, or 100% conditioned media from MCF-7 breast cancer cells with FCS 1% for 24 or 48 h or in media alone. The coverslips were placed on a Harvard Peltier plate and perfused with Krebs buffer (NaCl 126 mM, KCl 2.5 mM, NaHCO<sub>3</sub> 25 mM, NaH<sub>2</sub>PO<sub>4</sub> 1.2 mM, MgCl<sub>2</sub> 1.2 mM, and CaCl<sub>2</sub> 2.5 mM), pH 7.4 at 4°C to avoid receptor internalisation, as our probes are agonists. Dermorphin<sub>ATTO488</sub> (fluorescent green probe for MOP) or N/OFQ<sub>ATTO594</sub> (fluorescent red probe for NOP) was added to the coverslips at a concentration of 100 nM for 3 min, washed with ice-cold Krebs buffer for 2 min, and then imaged. Images were acquired using a Nikon (Amstelveen, The Netherlands) C1Si confocal microscope (60× immersion oil) at the desired wavelength, as described previously.<sup>22,23</sup> Specific binding for Dermorphin<sub>ATTO488</sub> was determined using naloxone (25 μM) or unlabelled dermorphin (25 μM) and for N/OFQ<sub>ATTO594</sub> with the NOP antagonist SB-612111 (25 μM).<sup>24</sup> Cells were pre-incubated with the ligands for 5 min before adding Dermorphin<sub>ATTO488</sub> 100 nM or N/OFQ<sub>ATTO594</sub> 100 nM. Because of some issues with imaging, we also included lipopolysaccharide (LPS; 100 ng ml<sup>-1</sup>) as a positive control for induction of NOP expression, as reported in a previous study.<sup>18</sup> Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI).

### Wound healing assay to measure NOP and MOP receptor activity

HUVECs were cultured in gelatine 0.2%-coated 12-well plates (CELLSTAR™ Greiner Bio-One, Thermo-Fisher, UK). When confluent, cells were washed once with PBS and incubated for 2 h in serum-free media 199 in the presence or absence of VEGF 10 ng ml<sup>-1</sup> and FGF 10 ng ml<sup>-1</sup> or 100% MCF-7 conditioned media with FCS 1%. A vertical scratch (wound) is formed in the middle of the well using a p200 pipette tip. Cells were washed three times with PBS to remove any floating cells that might attach to the edges of the wound. Cells were then incubated in various combinations with morphine 100 nM or N/OFQ 100 nM, VEGF 10 ng ml<sup>-1</sup> plus FGF 10 ng ml<sup>-1</sup>, or 100% MCF-7 conditioned media with FCS 1% alongside untreated control samples. The plate was transferred to an environmental chamber with CO<sub>2</sub> and temperature control that is installed on a Nikon ECLIPSE Ti-E microscope. Images of every scratch were taken every 30 min for a total of 17–18 h using the Nikon Perfect Focus System to keep the samples in focus during long live imaging experiments; wound closure was assessed with analysis to the 14-h time point. Images were analysed by Fiji using a plugin.<sup>25</sup>

### Data analysis

In qPCR assays, all 32 patient cell lines were used. Data are expressed as C<sub>T</sub> (cycle threshold) and relative to the geometric mean of the two housekeeper genes (ELF2B1 and B2M), ΔC<sub>T</sub>. Receptor expression for MOP and NOP using Dermorphin<sub>ATTO488</sub> and N/OFQ<sub>ATTO594</sub> used six patient cell lines in various combinations to a minimum of six, and representative data sets are depicted. For the wound healing assays, 14 patient cell lines

were used. Percentage of wound closure was analysed according to the following equation: wound closure % = [(T<sub>0</sub> - T<sub>n</sub>) / T<sub>0</sub>] × 100%, where T<sub>0</sub> is the initial wound area, and T<sub>n</sub> is the wound area after n hours of the initial scratch. For both qPCR and wound healing assays, data were analysed using analysis of variance with Dunn's multiple comparison test. P-values <0.05 were considered statistically significant.

## Results

### qPCR

Messenger RNA extracted from 32 patient HUVEC cell lines was probed for MOP and NOP expression. MOP mRNA was not detected in any sample tested. NOP mRNA was present in all samples with a mean ΔC<sub>T</sub> of 6.06 (Table 1). Incubation with 50% or 75% (one sample was lost in preparation; n=31) conditioned media from MCF-7 breast cancer cells for 24 h produced mean ΔC<sub>T</sub> values of 6.29 and 5.67, respectively; these did not differ from control. The cells did not survive when incubated with MCF-7 conditioned medium for 48 h. Incubation with VEGF 10 ng ml<sup>-1</sup> and FGF 10 ng ml<sup>-1</sup> for 24 or 48 h produced a mean ΔC<sub>T</sub> of 7.35 and 6.25, respectively; these did not differ from control. There was no statistically significant tumour or angiogenic signal (Table 1).

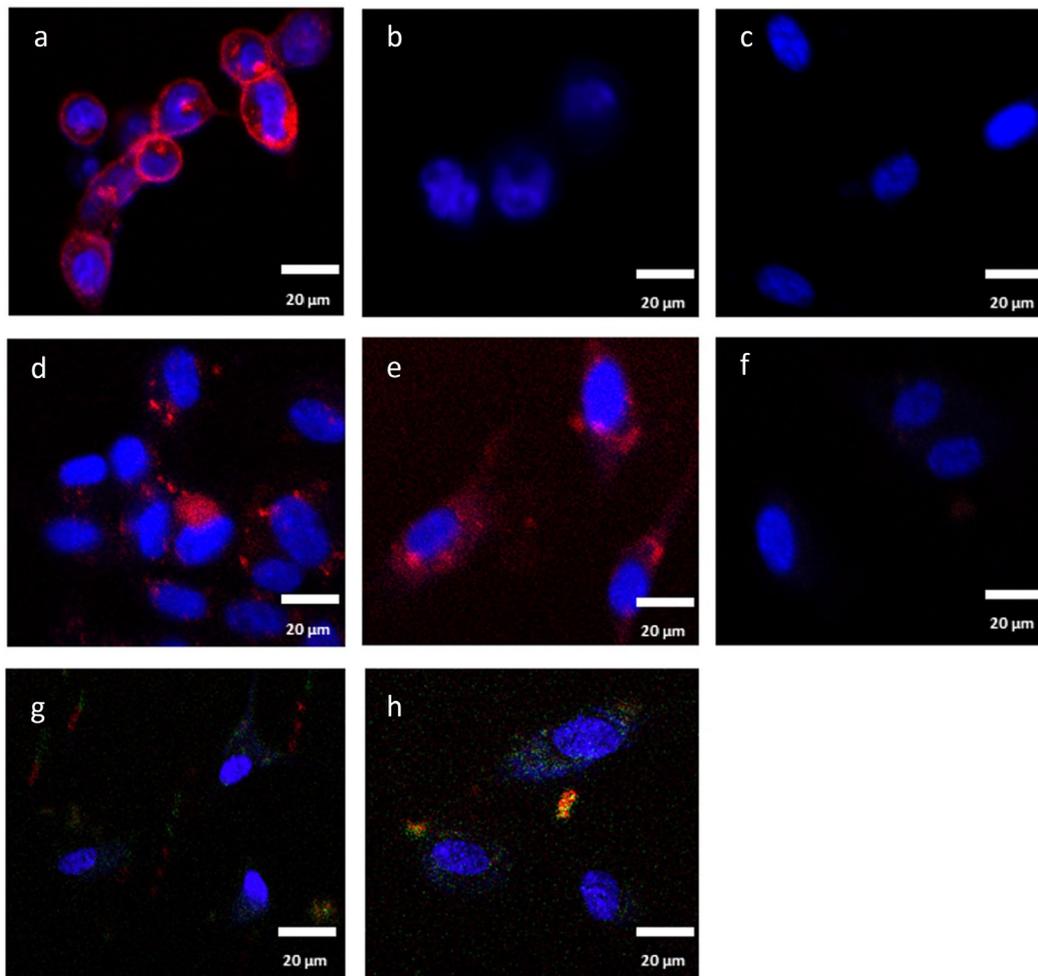
### Live-cell imaging

To examine the presence of MOP and NOP receptors on the cell surface of HUVECs, the fluorescent probes N/OFQ<sub>ATTO594</sub> (red) and Dermorphin<sub>ATTO488</sub> (green), which are selective for NOP and MOP, respectively, were used.<sup>22, 23</sup> In all images, nuclei were labelled with DAPI (blue).

As a positive control for NOP receptors, N/OFQ<sub>ATTO594</sub> bound to recombinant NOP receptors expressed in HEK cells (Fig. 1a), and this was displaced by the NOP receptor antagonist SB-612111, confirming selectivity and surface binding, as the antagonist will not cross the membrane (Fig. 1b). Despite possessing NOP receptor mRNA, HUVECs did not express surface

**Table 1** NOP mRNA expression in HUVECs. The presence of NOP receptor mRNA was detected in HUVECs treated for 24 h with 50% or 75% conditioned media from MCF-7 breast cancer cells or VEGF/FGF 10 ng ml<sup>-1</sup> for 24 or 48 h. To determine ΔC<sub>T</sub>, the geometric mean of the housekeeper genes ELF2B1 and B2M was used. Data are presented as mean [standard error of the mean] of 32 assays (in 32 individual cell lines) for control and treated samples and 31 for 75% MCF-7 cells. There was no MOP receptor mRNA detected and in any cell line, and there was no amplification in any non-template control. There were no statistically significant differences (analysis of variance) amongst the groups. FGF, fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; MCF-7, Michigan Cancer Foundation-7; NOP, naloxone-insensitive Nociceptin/Orphanin FQ peptide receptor; VEGF, vascular endothelial growth factor.

Condition	HK-C <sub>T</sub> (GEOM)	NOP-C <sub>T</sub>	ΔC <sub>T</sub>
Control	25.43 [0.35]	32.03 [0.49]	6.60 [0.55]
MCF-7 media 50% (24h)	25.31 [0.47]	31.60 [0.49]	6.29 [0.40]
MCF-7 media 75% (24h)	25.78 [0.40]	31.45 [0.45]	5.67 [0.44]
VEGF/FGF 10ng ml <sup>-1</sup> (24h)	24.38 [0.36]	31.73 [0.46]	7.35 [0.53]
VEGF/FGF 10ng ml <sup>-1</sup> (48h)	25.22 [0.53]	31.47 [0.47]	6.25 [0.36]

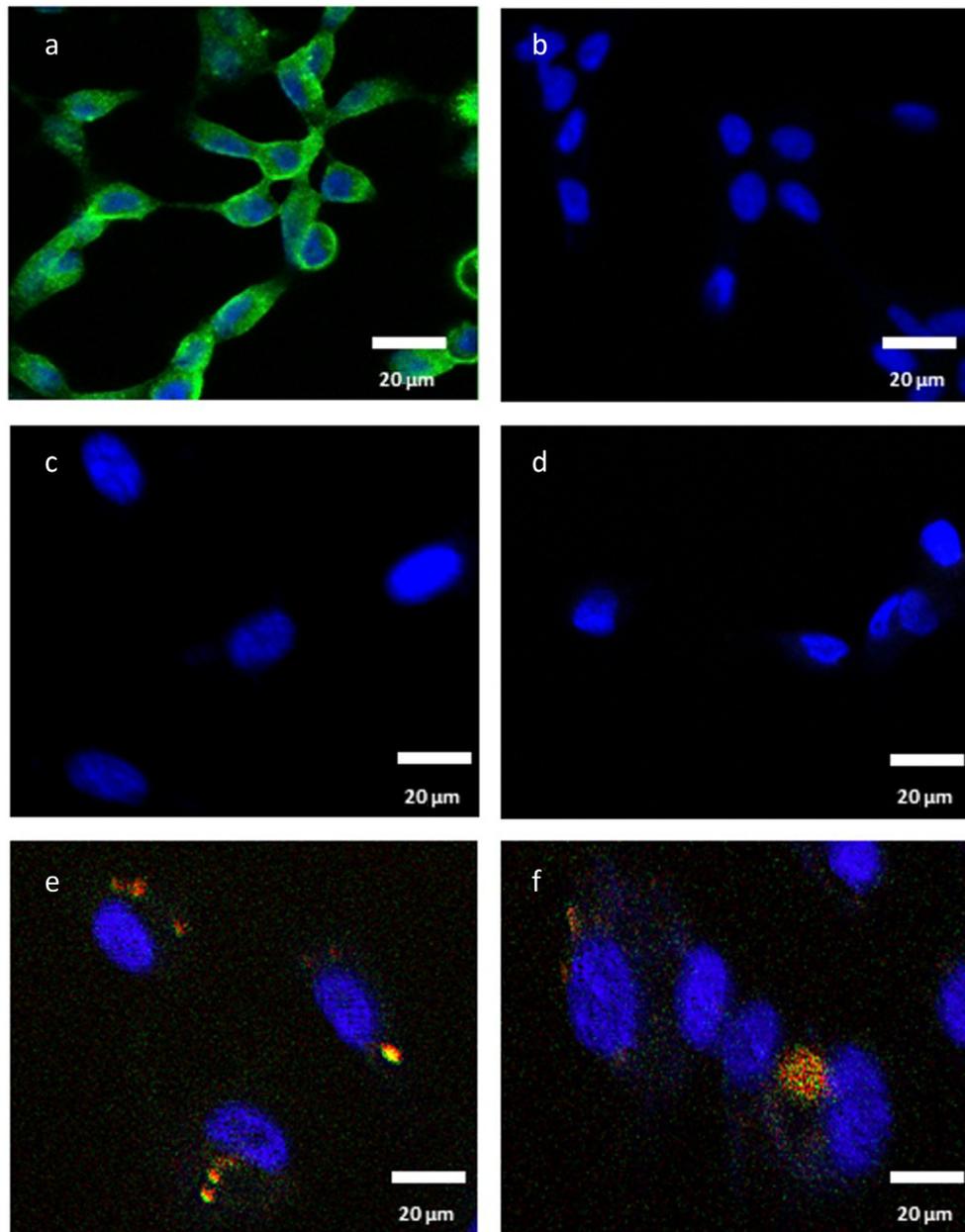


**Fig 1.** Probing cell surface NOP receptor expression. Binding of N/OFQ<sub>ATTO594</sub> (100 nM; red) to NOP receptors on the surface of HEK<sub>hNOP</sub> in the (a) absence and (b) presence of the NOP antagonist SB-612111 as a positive control in a high expression system. (c) Untreated HUVECs did not bind N/OFQ<sub>ATTO594</sub>. As a further induction positive control,<sup>18</sup> HUVECs treated with LPS (100 ng ml<sup>-1</sup>) for (d) 24 h and (e) 48 h displayed N/OFQ<sub>ATTO594</sub> binding that could be displaced by SB-612111 (48-h data shown in f). In HUVECs treated with (g) MCF-7 conditioned media (100%+FCS 1%) or (h) VEGF/FGF (10 ng ml<sup>-1</sup>) for 24 h, images were consistently grainy; there was no clear surface N/OFQ<sub>ATTO594</sub> (red) binding. Nuclei are stained blue with DAPI. Data are representative from *n*=6 cell lines. DAPI, 4',6-diamidino-2-phenylindole; FCS, fetal calf serum; FGF, fibroblast growth factor; HEK, human embryonic kidney; HUVEC, human umbilical vein endothelial cell; LPS, lipopolysaccharide; MCF-7, Michigan Cancer Foundation-7; N/OFQ, Nociceptin/Orphanin FQ; NOP, naloxone-insensitive N/OFQ peptide receptor; VEGF, vascular endothelial growth factor.

receptors, as N/OFQ<sub>ATTO594</sub> did not bind (Fig. 1c); this is consistent with our previous report.<sup>18</sup> As a further control for induction of expression, treatment with LPS (100 ng ml<sup>-1</sup>) for 24 h (Fig. 1d) or 48 h (Fig. 1e) increased NOP receptor translation such that the probe was now bound; this binding was also blocked by SB-612111 (Fig. 1f; 48 h data shown). In an extensive series of experiments, treatment with MCF-7 conditioned media for 24 h (Fig. 1g) or VEGF/FGF for 24 h (Fig. 1h) produced highly granular images with bleed into various confocal wavelengths. These images were not affected by NOP receptor block with SB-612111. The absence of clear surface N/OFQ<sub>ATTO594</sub> (red) binding coupled with no antagonist effects indicated that NOP receptor mRNA is not translated into receptor protein in the presence of angiogenic stimuli.

As a positive control for MOP receptor expression, Dermorphin<sub>ATTO488</sub> bound to recombinant MOP receptors

expressed in HEK cells (Fig. 2a), and this was displaced by the opioid antagonist naloxone, confirming classical opioid binding and cell surface location (Fig. 2b). Consistent with the lack of MOP receptor mRNA, HUVECs did not bind Dermorphin<sub>ATTO488</sub> in the absence (Fig. 2c) or presence (Fig. 2d) of naloxone. In an extensive series of experiments, treatment with MCF-7 conditioned media for up to 24 h (Fig. 2e) or VEGF/FGF for up to 48 h (Fig. 2f) produced highly granular images with bleed into various confocal wavelengths. These images were not affected by MOP receptor block with either naloxone or in a limited series of experiments with unlabelled Dermorphin. The lack of clear surface Dermorphin<sub>ATTO488</sub> (green) binding, with no antagonist effects coupled with a lack of MOP receptor mRNA, indicates that HUVECs do not express MOP receptors.

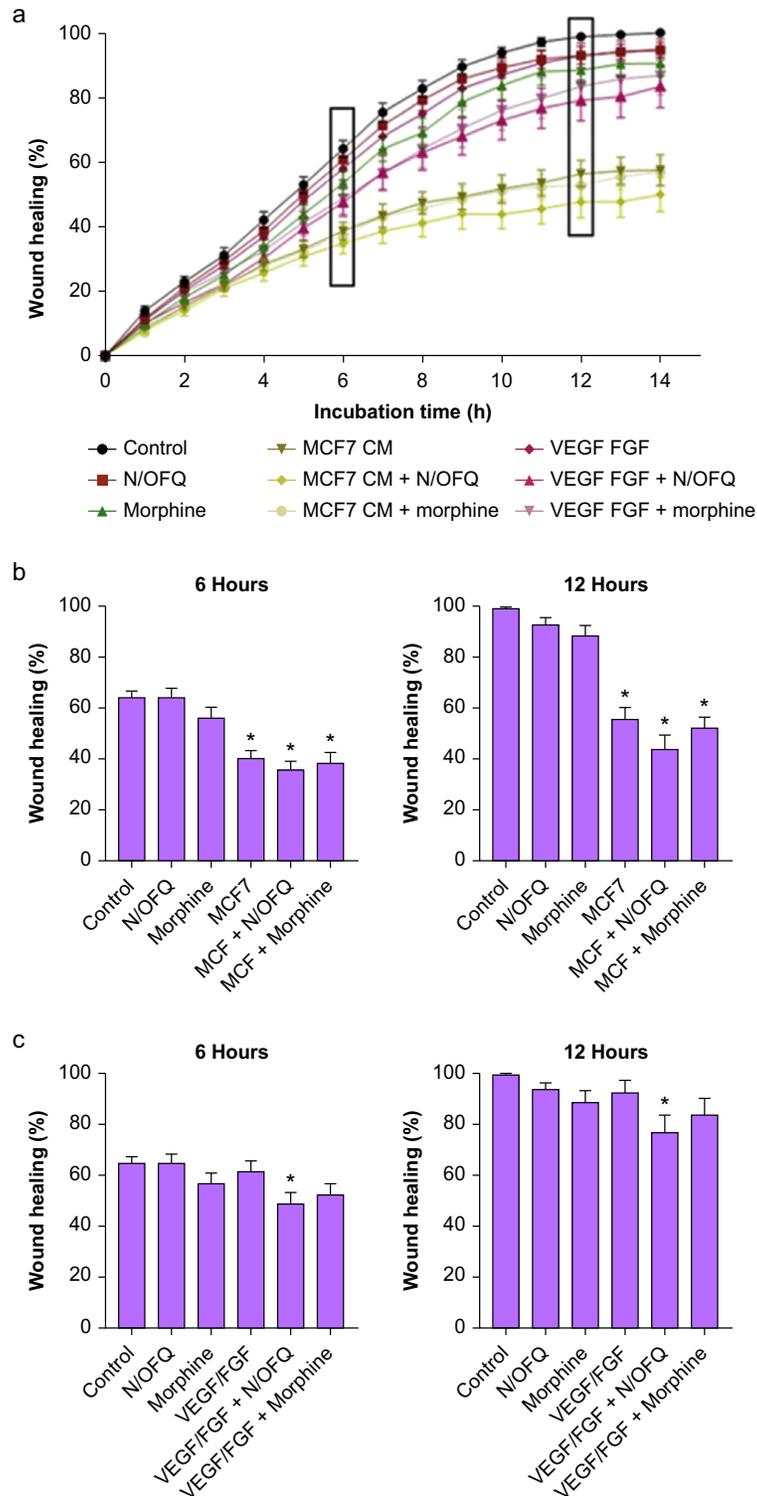


**Fig 2.** Probing cell surface MOP receptor expression. Binding of Dermorphin<sub>ATTO488</sub> (100 nM; green) to MOP receptors on the surface of HEK<sub>hMOP</sub> in the (a) absence and (b) presence of the opioid antagonist naloxone (25  $\mu$ M) as a positive control in a high expression system. HUVECs treated with Dermorphin<sub>ATTO488</sub> (100 nM) failed to bind the fluorescent probe in the (c) absence or (d) presence of naloxone. Images for Dermorphin<sub>ATTO488</sub> were consistently grainy in HUVECs treated with (e) MCF-7 conditioned media (100%+FCS 1%) or (f) VEGF/FGF for 24 h. Nuclei are stained blue with DAPI. Data are representative from  $n=6$  cell lines. DAPI, 4',6-diamidino-2-phenylindole; FCS, fetal calf serum; FGF, fibroblast growth factor; HEK, human embryonic kidney; HUVEC, human umbilical vein endothelial cell; MCF-7, Michigan Cancer Foundation-7; VEGF, vascular endothelial growth factor.

As the angiogenic stimuli consistently produced uncharacteristic grainy images, we examined the fluorescence properties of MCF-7 conditioned media and VEGF/FGF at the concentrations used in the perfusion system. There was no evidence of fluorescence in either red or green channel.

### Wound healing

As a surrogate for the early stages of angiogenesis (cell migration), we have measured scratch-wound healing in HUVECs. Cell lines from 14 individual patients were examined. There was a time-dependent closure of the wound in all



**Fig 3.** Effects of N/OFQ and morphine on wound healing. HUVECs were cultured in 12-well plates; a scratch was created in each well, which was then incubated with media from MCF-7 breast cancer cells or VEGF/FGF. N/OFQ (100 nM) or morphine (100 nM) was included as indicated. (a) A 14-h time course. (b) Data at 6 and 12 h are shown for cells exposed to 100% MCF-7 conditioned medium + FCS 1% and (c) for VEGF/FGF 10 ng ml<sup>-1</sup>. Data are mean [standard error of the mean] (n=14). (a) Full time courses were significant by ANOVA (P<0.05). (b) and (c) Data were significant by ANOVA (P<0.05) and \*P<0.05 reduced compared with control (Dunn's multiple comparison test). Representative raw images are shown in the Supplementary material. ANOVA, analysis of variance; FCS, fetal calf serum; FGF, fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; MCF-7, Michigan Cancer Foundation-7; N/OFQ, Nociceptin/Orphanin FQ; VEGF, vascular endothelial growth factor.

conditions tested (Fig. 3a). The data were analysed further at two time points, 6 and 12 h, with the latter representing full closure under control conditions. At both 6 and 12 h, N/OFQ (100 nM) or morphine (100 nM) alone failed to influence wound healing. Treatment with MCF-7 conditioned medium delayed and reduced wound healing, and this was unaffected by N/OFQ (100 nM) or morphine (100 nM) (Fig. 3b). Treatment with VEGF/FGF did not influence wound healing; there was a small but statistically significant delay in healing with VEGF/FGF in the presence of N/OFQ when compared with control but not with VEGF/FGF alone (Fig. 3c).

## Discussion

The effects of classical opioids on angiogenesis are controversial,<sup>6,14</sup> and there are currently no data for the non-classical N/OFQ receptor, NOP. We have used primary cultured HUVECs of low passage as models for the angiogenic process. Early-passage primary cultures offer the advantage of limited passage-related de-differentiation and therefore more closely mimic the *in vivo* situation, accepting that this is a monolayer culture rather than a vessel structure. As angiogenic stimuli, we have used VEGF/FGF and conditioned medium from the MCF-7 breast cancer cell line; the latter representing a possible metastatic re-seeding signal. We have shown that HUVECs express mRNA for the NOP receptor but not the MOP receptor, and that the angiogenic stimuli do not influence the levels of mRNA. With respect to MOP receptors, the absence of mRNA predicts the absence of receptor protein, and hence lack of effect on migration (wound healing). NOP receptor mRNA is present, but this is not translated to receptor, as there was no N/OFQ<sub>ATTO594</sub> binding; the lack of cell surface NOP receptors did not influence migration. MCF-7 medium alone reduced wound healing, and this would potentially suggest an anti-angiogenic response to this specific tumour microenvironment.

In a previous study using HUVECs, we have reported NOP receptor mRNA. In this study, we examined an *in vitro* septic stimulus of LPS and peptidoglycan G. These stimuli led to a translation of NOP receptor mRNA into protein as measured by increased N/OFQ<sub>ATTO594</sub> binding. These receptors were functionally active, as they activated the MAP kinase pathway leading to phosphorylation of extracellular signal-regulated kinase (ERK1/2).<sup>18</sup> As a positive control for induction of expression in the present study, we have included LPS in our confocal experiments and exactly replicated this increased mRNA translation into receptor protein.<sup>18</sup>

For the detection of NOP and MOP receptors on the cell surface, the NOP-specific N/OFQ<sub>ATTO594</sub> and the MOP-selective Dermorphin<sub>ATTO488</sub> fluorescent probes were used.<sup>22,23</sup> As a further positive control for NOP receptors, we used HEK cells transfected with and expressing high levels of the human NOP receptor.<sup>23</sup> As a control for MOP receptors, we used HEK cells transfected with and expressing high levels of the human MOP receptor.<sup>22</sup> The absence of MOP receptor mRNA is consistent with the absence of Dermorphin<sub>ATTO488</sub> binding. We had anticipated an angiogenic stimulus-driven translation of NOP mRNA into protein, but there was no N/OFQ<sub>ATTO594</sub> binding. We have used these probes in recombinant cell lines,<sup>22,23</sup> rat brain tissue,<sup>19</sup> and immune cells,<sup>23</sup> and we have consistently observed clean confocal binding signals, even when treated with pseudo-septic stimuli.<sup>18</sup> In the present study where we have observed clean binding data with transfected HEK cells and in repeats of LPS treatment, the use of angiogenic stimuli

(MCF-7 conditioned medium and VEGF/FGF) produced variably grainy images. HUVECs did not survive beyond 24 h with MCF-7 conditioned medium, and it is tempting to suggest that this and possibly VEGF/FGF long-term treatment is cytotoxic with toxicity affecting cellular fluorescence properties. We do not believe this fluorescence was to the receptor. In support of this, cells pre-incubated with high concentrations (25 µM) of the antagonists SB-612111<sup>24</sup> or naloxone to fully occupy any receptor-binding sites did not affect the quality of any N/OFQ<sub>ATTO594</sub> or Dermorphin<sub>ATTO488</sub> images. Moreover, MCF-7 conditioned medium and VEGF/FGF did not fluoresce at the wavelengths used for N/OFQ<sub>ATTO594</sub> or Dermorphin<sub>ATTO488</sub>. Collectively, these data confirm no surface expression of either the NOP or MOP receptor in native or angiogenic stimuli challenged HUVECs.

In an early study from Granata and colleagues,<sup>26</sup> HUVECs were used to explore a role for the NOP receptor (termed OP4 in their paper). The presence of OP4 receptor protein was shown in immunoblots using anti-OP4 antibodies; a 64 kDa band was identified, which was also present in hippocampus and capillaries. Expression was further inferred by the activation of ERK1/2 in western blotting. In addition, 1 µM N/OFQ led to a rapid increase in ERK1/2 phosphorylation at 5 min, returning towards baseline by 20 min. The origin of these HUVECs was not described and appears to be commercial. As noted, these cells de-differentiate with time in culture and passage number; the number of lines used was not described. It is difficult to reconcile these data, from an indeterminate number of lines, with our own. Of note, in our study, we were able to stimulate mRNA translation into protein using LPS,<sup>18</sup> again suggesting the lack of protein is not simply methodological.

As a potential downstream functional assay for angiogenesis and the effects of opioids, we selected the wound healing assay.<sup>27</sup> This assay probes the early phase of angiogenesis; migration. In this assay, wound closure for the untreated control samples occurred in 10–12 h of incubation. Effects were further interrogated at 6 and 12 h. The rate of closure was not adversely affected by the defined angiogenic stimuli VEGF/FGF with no difference from control. With the absence of cell surface receptors, there was no major effect of either N/OFQ or morphine; both were used at concentrations of 100 nM that would occupy and activate both NOP and MOP receptors, respectively, had they been present. The combination of VEGF/FGF and N/OFQ produced a small reduction in wound healing compared with control; we have no explanation for this small but statistically significant change, although apoptosis is possible. In contrast, exposure to conditioned media from MCF-7 breast cancer cells markedly reduced migration and hence wound closure. Interestingly, in most cell lines used, areas devoid of cells could be seen beyond the scratch, suggesting that cells were dying during the assay (Supplementary image). Lee and Kang<sup>28</sup> examined migration of endothelial colony-forming cells in response to a conditioned medium from MDA-MB-231 or MCF-7 cells, and in agreement with our data showed reduced migration with MCF-7 conditioned medium. The authors noted that MCF-7 cells secrete less angiogenic stimuli than MDA-MB-231.<sup>28</sup> However, a reduction against control observed in the present study suggests that MCF-7 cells may secrete an anti-migratory substance.

The effects of morphine on angiogenesis are controversial with stimulatory and inhibitory actions depending on the opioid receptor type, opioid concentration, and route of

application.<sup>6,14</sup> An interaction between opioids and VEGF has been suggested based on the findings that in endothelial cells, VEGF increases MOP receptor expression.<sup>4</sup> In our hands and with low-passage HUVECs, no mRNA encoding for MOP receptors was detected, and this was not influenced by VEGF/FGF. The lack of MOP receptors *per se* does not exclude a role for morphine in the control of angiogenesis, as there is compelling evidence for naloxone-sensitive interaction with TLR4 receptors,<sup>29,30</sup> and HUVECs express this target for LPS.<sup>31</sup> Indeed, LPS acting at TLR4 is capable of translating NOP receptor mRNA into protein. However, in our assay, morphine did not affect migration and hence early angiogenesis; this lack of effect of morphine is consistent with some previously published data.<sup>14,32,33</sup>

Opioids are not used in isolation, rather in combination with a range of anaesthetic agents. In our study, we were interested in the effects of opioids alone, which can then be worked up to combinations with other agents. To reiterate, we were particularly interested in NOP, as this (along with MOP) is a target for the late-phase investigational drug cebranopadol.<sup>10</sup> In a paper from Luo and colleagues,<sup>34</sup> the effects of isoflurane on the malignant potential of the SK-OV3 ovarian cancer cell line were studied. The authors showed that isoflurane increased VEGF and angiopoietin expression in tumour cells; this was presumed to be released, and indeed, a medium from isoflurane-treated cells supported angiogenesis and enhanced wound healing. In our study, medium from MCF-7 cells reduced wound healing, and this may indicate tumour-specific differences. It should be borne in mind that this is a very crude assay for the early stages of angiogenesis.

Collectively, we did not identify cell surface MOP or NOP receptors on freshly isolated HUVECs, and this was unaffected by potential angiogenic stimuli.

## Authors' contributions

Obtained funding: DGL.  
Provision of primary human vascular tissues: SY.  
Preparation of primary vascular cultures: DGM, FA-J.  
Provision of fluorescent probes: RG, GC.  
Primary data collection: DG, SK, WM.  
Data analysis: DG, SK, WM, RG, GC, SY, DGL.  
Writing of article: all authors.  
Approval of final version: all authors.

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## Declarations of interest

DGL is a scientific adviser to Cellomatics, a small and medium-sized enterprise/contract research organisation, and chairs the board of the British Journal of Anaesthesia.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bjao.2022.100110>.

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