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Research

Immunoexpression of the relaxin receptor LGR7 in breast and uterine tissues of humans and primates

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Abstract

Background: The receptor for the peptide hormone relaxin has recently been identified as the heptahelical G-protein coupled receptor, LGR7. In order to generate molecular tools with which to characterize both *in vivo* and *in vitro* expression of this receptor in human and primate tissues, specific monotypic antibodies have been generated and applied to a preliminary analysis of human and primate female reproductive tissues.

Methods: Three peptide sequences were identified from the proposed open reading frame of the cloned LGR7 receptor gene, representing both extracellular and intracellular domains. Two to three rabbits were immunized for each epitope, and the resulting sera subjected to a systematic validation using cultured cells transiently transfected with a receptor-expressing gene construct, or appropriate control constructs.

Results: Human and monkey (marmoset, macaque) endometrium showed consistent and specific immunostaining in the stromal cells close to glands. Staining appeared to be more intense in the luteal phase of the cycle. Weak immunostaining was also evident in the endometrial epithelial cells of the marmoset. A myoma in one patient exhibited strong immunostaining in the circumscribing connective tissue. Uterine expression was supported by RT-PCR results from cultured primary endometrial and myometrial cells. Human breast tissue (healthy and tumors) consistently indicated specific immunostaining in the interstitial connective (stromal) tissue within the glands, but not in epithelial or myoepithelial cells, except in some tumors, where a few epithelial and tumor cells also showed weak epitope expression.

Conclusions: Using validated monotypic antibodies recognizing different epitopes of the LGR7 receptor, and from different immunized animals, and in different primate species, a consistent pattern of LGR7 expression was observed in the stromal (connective tissue) cells of the endometrium and breast, consistent also with the known physiology of the relaxin hormone.

Background

For many years the heterodimeric peptide hormone relaxin was considered only as a molecule involved in the periparturient widening of the pubic symphysis and softening of the cervix. More recently, it has been shown that relaxin, whose structure is similar to that of insulin, is also involved in endometrial differentiation associated with decidualization and embryo implantation [1,2]. Relaxin acts on primary cultures of endometrial stromal cells also *in vitro* to induce the morphological and gene expression changes, known as decidualization [1], which are the essential prerequisite for implantation to occur. In fact, relaxin in this context appears to be more effective even than progesterone [1]. In addition, altered levels of circulating relaxin, presumably of ovarian origin, are linked to early embryonic loss due to implantation failure [3,4], and it has been shown that luteinizing ovarian granulosa cells from IVF treatments produce higher relaxin levels in association with good pregnancy outcome [5]. Within the female reproductive system, relaxin has also been shown to suppress spontaneous contractions of the myometrium in the rat [6], and to modulate uterine connective tissue metabolism by regulating the expression of matrix metalloproteinases [7]. Most recently, it has also been shown to positively influence uterine angiogenesis, probably by the induction of local VEGF [8,9].

Relaxin is also known to be produced and have effects within the breast [10,11]. Both H1 and H2 forms of human relaxin have been shown to be synthesized by the epithelial and myoepithelial cells of the human breast [11]. Relaxin also influences growth parameters in breast cancer cell-lines, probably using local NO pathways [12,13], and recently, it has been shown that significantly higher concentrations of circulating relaxin are associated with the formation of breast cancer metastases [14]. Partly this appears to be due to an increased ability of breast cancer cells to invade extracellular matrix upon relaxin stimulation [15].

Two receptors responding to relaxin, LGR7 and LGR8, were cloned and described in 2002 [16,17]. These are novel members of the G-protein-coupled receptor superfamily, with a heptahelical transmembrane domain and a large glycosylated ectodomain, and are distantly related to the receptors for the glycoproteohormones, such as LH or FSH. In transfected cell systems, these receptors are shown to respond to relaxin by causing an elevation of intracellular cAMP, presumably through a G-protein-dependent activation of adenylate cyclase [16]. In human primary endometrial stromal cells, a similar elevation in cAMP is detected upon relaxin stimulation, but here inhibition of intracellular phosphodiesterases also appears to be playing an important role [18,19]. Interestingly, pharmacological studies show that for the human LGR8 both

relaxin and the recently identified peptide INSL3 (also called relaxin-like factor, RLF) can act as effective ligands, whereas LGR7 only responds to relaxin [17]. In rodents, the equivalent receptors respond exclusively to either relaxin (LGR7) or INSL3 (LGR8), with no overlap in ligand specificity [20]. Neither receptor is able to bind or respond to any of the other related peptide hormones, such as insulin, the IGF family, or the recently identifed INSL4 [16,17,20,21]. Very recently, two further novel receptors, GPCR135 and GPCR142, have also been characterized as responding to a relaxin-like molecule, namely relaxin 3 [22,23]. These receptors respond only very weakly to the porcine H2 relaxin homologue and appear not to be expressed in non-pregnant female reproductive tissues [22,23].

Studies on receptor expression and localization in tissues have, until now, relied upon in situ relaxin-binding, using either radioactively labelled or biotinylated ligand [e.g. [24-26]]. These methods mostly appear unable to yield the sensitivity or specificity which can identify precisely the cell types where the relaxin receptors are expressed, and would be unable to distinguish receptors from other proteins interacting with the hormone at high specificity (analogous to the IGF-binding proteins). Alternatively, primary cells can be prepared and subjected to Scatchard analysis. Where this has been carried out, for example for endometrial stromal cells, relaxin receptors are shown to be expressed at a low concentration (ca. 1000 molecules per cell), though they have a high affinity for the peptide ($K_d \sim 1$ nM) [27].

In order to gain a better appreciation of the expression of the LGR7 receptor protein in both normal and pathological human tissues, we have generated a series of monotypic polyclonal antibodies, using as immunogens peptides from within different regions of the human LGR7 protein sequence. These antibodies have been systematically validated, and applied for the first time here to human breast tissue, and human and primate uterus.

Methods and Materials
Generation of antibodies

Three different peptides were designed using the published human LGR7 sequence as basis. Two peptides (L7-1: CFKNYHDLQKLYLQN AND L7-2: MRKNKINHLNENTFAC) were derived from the large ectodomain in regions which from preliminary folding bioinformatics suggested the sequences to be relatively hydrophilic and exposed on the surface of the protein, and not masked by neighbouring glycosylations at putative Asn-X-Thr/Ser sites. A further peptide epitope (L7-3: CSAITATEIRNQVKKEM) was derived from the sequence of the third intracellular loop, in a region which has been shown to generate good antibodies against other G-protein coupled

receptors, for example, the oxytocin receptor [28]. All peptide sequences were checked for uniqueness by searching the international databases. The peptides were then chemically synthesized and coupled via N- or C-terminal cysteine residues to keyhole limpet hemocyanin, before using as immunogens in rabbits, following a conventional immunization protocol [29]. Peptide production and immunization was carried out by a commercial company (Pineda Antibody Services, Berlin, Germany). All rabbits were checked for non-specific binding of sera prior to immunization, and pre-immune sera were collected from all animals selected for immunization. Animals were bled following the second boost, and these sera used for all subsequent studies.

Cloning and transfection of human LGR7 and LGR8 expression constructs

Full-length cDNA constructs for human LGR7 and LGR8 were kindly provided by Professor Aaron Hsueh (Stanford University, CA). These were inserted into the pcDNA3.1-Zeo expression vector (Invitrogen, San Diego, CA) driven by a CMV viral promoter. In order to improve protein expression the natural signal sequences of the two receptors had been replaced by that of the human prolactin precursor polypeptide. In order to validate the specificity of the antibodies generated, the LGR7 and LGR8 expression constructs were transiently transfected into HEK293T cells, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, at a DNA concentration of 4 μg expression plasmid (encoding LGR7, LGR8 or the orphan G-protein coupled receptor, HE6) and 0.2 μg pEGFP-N1 (encoding enhanced green fluorescent protein; BD Biosciences, Heidelberg, Germany) per well. Cells were grown in 6-well plates (Nunc, Roskilde, Denmark) at a density of 10^6 cells per well in DMEM with phenol red (Sigma, Taufkirchen, Germany), 10% FCS Gold (PAA Laboratories, Linz, Austria), 2 mM L-Glutamine (Gibco Paisley, UK), without antibiotics. Transfection was performed after 24 h in serum-free OPTI-MEM medium (Gibco). After a further 24 hours, the transfected cells were seeded into T25 flasks (Nunc), and after overnight incubation were trypsinized, washed three times in PBS (Sigma) at 4°C, and transferred to cytospin chambers at a concentration of 50000 cells per chamber. After centrifugation at 800 rpm for 5 mins in a cytospin centrifuge, media was removed and the cells air-dried, and then fixed in 3% (w/v) paraformaldehyde for 10 mins, followed by 3 washes of 5 min each in PBS, and then permeabilization in 0.1% (v/v) Triton X-100 in PBS for 5 min. After further washing twice for 5 min in PBS, the cytospin preparations were subjected to immunohistochemistry. In order to validate the new antibodies generated, immune and the respective pre-immune sera from the same animals were diluted 1:4000, and applied to the permeablized cytospin preparations. Specific immunoreactions were visualized using

the double PAP-ABC combination technique (see below) as previously described [30], but without additional nuclear counterstaining. As a further control, also an expression construct encoding HE6 (generous gift from Dr. C. Osterhoff, Hamburg, Germany), was similarly transfected into HEK293T cells. This receptor belongs to a discrete subfamily of heptahelical domain receptors from LGR7, though also possesses a large glycosylated ectodomain [31,32]. The internal control transfections using the EGFP expression construct all indicated a high percentage (>60%) of cells transfected for all construct combinations (not shown).

Immunohistochemistry of human and primate tissues

Human tissues from four patients were collected in observance of the Helsinki Declaration, and with the authorization of the local ethical committee. Uterine tissue from two cynomolgus macaques (secretory phase) was a generous gift from Dr UF Habenicht (Schering AG, Berlin, Germany) and was also obtained with full ethical approval from the appropriate authority. Marmoset monkey tissues (five breeding females, 3–6 years old) were collected, with the approval of the local ethics commission on animal experiments, from a breeding colony at the German Primate Center in Göttingen, Germany, as previously described [4,33]. Tissues were immersion-fixed overnight in buffered 10% (w/v) formaldehyde (human tissues), in 5% buffered formaldehyde for 3 h (marmosets), or in Bouin's solution overnight (macaque), before passaging through ascending ethanols to be embedded in paraffin. Human and macaque tissues were cut to 5–7 μm, and processed for immunohistochemistry as previously described [30], using the PAP(peroxidase-anti-peroxidase)-ABC(avidin-biotin-complex) combination method. Briefly, after passing through xylol and descending ethanols, entparafinized sections were washed 2 × 10 min in TBS (Tris-buffered saline; 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4), then endogenous peroxidase was suppressed by incubation in 3% (v/v) H_2O_2 for 45 min at room temperature (RT). Sections were then blocked with 10% (v/v) normal goat serum for 1 h at RT. After brief rinsing in TBS, sections were incubated with the primary antiserum, diluted 1:4000 (L7-1, L7-2) or 1:5000 (L7-3) in antibody dilution buffer (ADB; TBS containing 2% (v/v) normal goat serum plus 0.05% (w/v) bovine serum albumin), overnight at 4°C. After rinsing 3 × 5 min in TBS, sections were then incubated with biotinylated goat-anti-rabbit IgG, diluted 1:500 in ADB for 1 h at RT. After 3 × 5 min rinsing in TBS, sections were then incubated in rabbit PAP-complex (Dianova, Hamburg, Germany), again diluted 1:500 in ADB, for 1 h at RT. After 3 × 5 min rinsing in TBS, these last two incubation steps were repeated, this time for only 30 min per step. After further brief rinsing in TBS, sections were then incubated with ABC-complex (Vector Laboratories, Burlingame, USA) for 1 h at RT.

Specific signals were detected using DAB (Sigma) as chromogen, stopping the reaction by rapidly rinsing in tapwater, followed by conventional haemalaun counterstaining. Sections were then coverslipped in aqueous mounting medium. Marmoset tissues used the complete ABC-complex staining system (Vector Laboratories) as recommended by the manufacturer with AEC as chromogen. As controls for immunohistochemical specificity, all sections were treated in parallel with both immune sera and the pre-immune sera from the same animals at the same dilutions. All immunohistochemical analyses were completely repeated at least once for all tissues, with identical results. As a further control, some sections were additionally exposed to diluted primary antisera which had been subject to a preincubation for 2 h at room temperature with pure keyhole limpet hemocyanin (Sigma, Deisenhofen, Germany) in TBS at a concentration of 10 μg/ml.

RT-PCR analysis for LGR7 gene transcripts

In order to provide some confirmatory evidence of LGR7 expression at the level of the gene transcript, a series of RT-PCR experiments were carried out on primary cells derived from human endometrial tissue obtained at hysterectomy for non-endocrine complaints. Procedures were approved by the local ethical committee and were in accord with the Helsinki Declaration on the scientific use of human tissues. Primary cultures of endometrial stromal cells and myometrial cells were prepared, as described previously [18,34,35]. Crude glandular epithelial cells were prepared following the enzymatic removal of stromal cells, followed by differential centrifugation, essentially according to the procedure of Bracken et al. [36] for the guinea-pig. Cultures were grown in basic media (BM) consisting of a 1:1 mixture of Ham's-F12:DMEM supplemented with 4 mM glutamine, 20 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin for 5–6 days until reaching confluency. For myometrial and stromal cells, BM was supplemented with 10% fetal calf serum (FCS) that had been depleted of steroids by treatment with dextran-coated charcoal, 1 μg/ml insulin and 10^{-9}M 17β-oestradiol (Sigma, Deisenhofen, Germany); for epithelial cells 5% FCS was used. This methodology was shown to produce stromal and epithelial cell cultures of >95% purity based on morphology and immunohistochemistical staining for vimentin, desmin, or cytokeratin (R.T. and Y.P, unpublished). The myometrial tissue used for cell culture was completely free of all endometrial contamination. Where indicated, cells were incubated or not with 2.5×10^{-7}M medroxyprogesterone acetate (MPA; Sigma), or a similar concentration of progesterone (Sigma), and/or 100 ng/ml pure porcine relaxin (courtesy Dr. O.D. Sherwood; University of Illinois, Urbana-Champain, IL). Cultures were submitted to differential trypsinization and attachment for further purification and subculture before

RNA was extracted for the RT-PCR analyses using RNeasy kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For RT-PCR 5 μg total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) exactly as recommended by the producer. Before each PCR reaction, samples were denatured at 95°C for 5 min. The amplification profile for LGR7 specific PCR reactions with primers #176, #177, #178, #180, #181, #189 and #191 consisted of denaturation at 94°C for 45 seconds, annealing at 54°C for 45 seconds and extension at 72°C for 2 min, followed by a final extension for 10 min at 72°C. For the LGR7 specific primers #174 and #178, and for GAPD (glyceraldehyde-3-phosphate dehydrogenase) specific primers #150 and #151 PCR conditions were modified to denaturation at 94°C for 45 seconds, annealing at 52°C for 1 min and extension at 72°C for 1 min. hPRL, IGF-BP1 and GAPD (primers #400, #401) transcripts were amplified according to Bartsch et al. [19]. Each reaction included 100 pmol of each primer pair as listed in Table 1. The identity of all PCR products was confirmed by DNA sequencing.

Results

Production and validation of polyclonal anti-LGR7 antibodies

Polyclonal antisera were produced in rabbits against three discrete peptide epitopes from the same polypeptide molecule which represents the principal receptor for the peptide hormone relaxin, LGR7. For each epitope 2–3 different animals were immunized, each of which had had sera checked prior to immunization for low background non-specific immunostaining. Therefore, as controls for the validity of the antibodies, we are looking for positive immunostaining which agrees in cell-type specificity for all three epitopes from the same protein molecule, and which further agrees between different animals for the same epitope. As a second control system, all antisera are compared directly on parallel sections with the pre-immune sera taken from the same animals and used at the same dilution. Thirdly, it is possible that some antibodies will be generated against the keyhole limpet hemocyanin used as hapten, although this molecule is not expressed in any mammalian cell. Nevertheless, there remains the possibility that such antibodies could recognize a cell-specific mimetic epitope. In order to check for this, in further control experiments, appropriately diluted antisera were pre-incubated with pure keyhole limpet hemocyanin to saturate any such antibodies, before presenting them to the tissue sections.

As principal positive control system, cytospin cultures of transiently transfected HEC293T cells were subjected to immunostaining under conditions identical to those used for the tissue sections. Cells were transfected either with a

Table 1: Oligonucleotides used for the RT-PCR reactions.

Transcript	Number	Orientation	Sequence	Expected Size
LGR7	#174	forward	5'-TCAACACATGCCAAGACTAC-3'	522 bp
LGR7	#176	reverse	5'-GCTTCATTAACTCAGGTGGCATCT-3'	(see Fig. 4C)
LGR7	#177	reverse	5'-CAAAAACACCCGGCTTCAGGA-3'	
LGR7	#178	reverse	5'-TGCAGATACAACCCAGACA-3'	
LGR7	#180	forward	5'-ATGACATCTGGTTCTGTCTTC-3'	
LGR7	#181	reverse	5'-TCATGAATAGGAATTGAGTCTCG-3'	
LGR7	#189	forward	5'-CGTGTGTGTAAAGAAGGAG-3'	
LGR7	#191	forward	5'-TGTGAGCTGTATGCGATTC-3'	
GAPD	#150	forward	5'-TCAGCAATGCCTCCTGCAC-3'	277 bp
GAPD	#151	reverse	5'-CTGCTTCACCACCTTCTTG-3'	
GAPD	#400	forward	5'-GCATCCTGGGCTACACTGAG-3'	140 bp
GAPD	#401	reverse	5'-ACCACCTGTTGCTGTAGCC-3'	
PRL		forward	5'-GACAGAGACACCAAGAAGAATCGGAACATA-3'	875 bp
PRL		reverse	5'-GCAATGGAACGGATCATTAAGGACCTTCTC-3'	
IGF-BP1		forward	5'-TGCTGCAGAGGCAGGGAGCCC-3'	378 bp
IGF-BP1		reverse	5'-AGGGATCCTCTTCCCATTCCA-3'	

human LGR7 expression plasmid, with an expression plasmid encoding the closely related human LGR8 receptor protein, or with a similar expression plasmid encoding the more distantly related heptahelical receptor, HE6. Transient transfection was deliberately used, so that within any one field of view both transfected (immunostained) and non-transfected (unstained) cells can be directly compared. Figure 1 shows that all three LGR7 epitopes have generated specific antibodies, which only recognize LGR7 protein (Fig. 1A,1D,1E,1F,1J,1J,1K,1L), and not the products of the transfected LGR8 (Fig. 1B,1G,1M) or HE6 genes (Fig. 1C,1I,1N). That the transfection for these gene constructs had properly functioned was tested by using independent antibodies specific for either LGR8 (Fig. 1H) or HE6 proteins (Fig. 1O). Similar results, though giving differing staining intensities and thus disclosing different antibody titers, were also obtained for all the different rabbit sera generated from each immunogen, whereby the L7-2 (Fig. 1D,1E,1F) and L7-3 (Fig. 1J,1K,1L) series were markedly better than those for L7-1.

Expression of LGR7 epitopes in primate and human endometrium

Tissue sections from the uterus of a cynomolgus macaque in the secretory (luteal) phase of the estrous cycle stained positively using all three LGR7 epitopes (Fig. 2A,2D,2G). Staining was consistently prominent in the endometrial stromal cells and to a varying extent also in the glandular epithelium. The negative controls using equivalent concentrations of pre-immune sera were consistently unstained (Fig. 2B,2E,2H). However, when antisera were preincubated with keyhole limpet hemocyanin (KLH), the epithelial staining observed in the absence of the hap-

ten (Fig. 2A,2D,2G, arrowheads) was markedly attenuated (Fig. 2C,2F,2I), whereas the stromal staining remained unaffected, implying that the former was likely to be due to an unspecific staining reaction. There was only very light and inconsistent staining in the myometrium (not shown).

Sections of human uterus gave essentially identical results, with prominent and specific immunostaining consistently only in the stromal cell compartment of the endometrium (Fig. 3A,3B). Negative controls using pre-immune sera (Fig. 3C,3D) or those using a preincubation step with KLH (not shown) indicated identical results to those with the macaque uterus, though in the human there was no detectable epithelial staining, even before KLH preincubation. Of interest was that, of three patient endometria examined, only one (Fig. 3) showed moderately intense staining comparable to that of the macaque. The other patients analysed showed only very weak or negligble staining (not shown). The morphological appearance of these tissues implied that these patients were in the proliferative or early secretory phase, whereas the positively immunostaining endometrium was from a patient in the mid secretory phase. Of interest was the observation, in the patient with significant LGR7 immunostaining, that small fibroid nodules exhibited intense immunoreactivity in the immediately surrounding non-myoid tissue (Fig. 3E,3F), which was absent in sections pretreated with the specific pre-immune serum (Fig. 3G,3H), and could not be suppressed by pre-treatment with KLH (not shown).

For further confirmation of LGR7 gene expression in the human uterus, RT-PCR analyses were carried out using
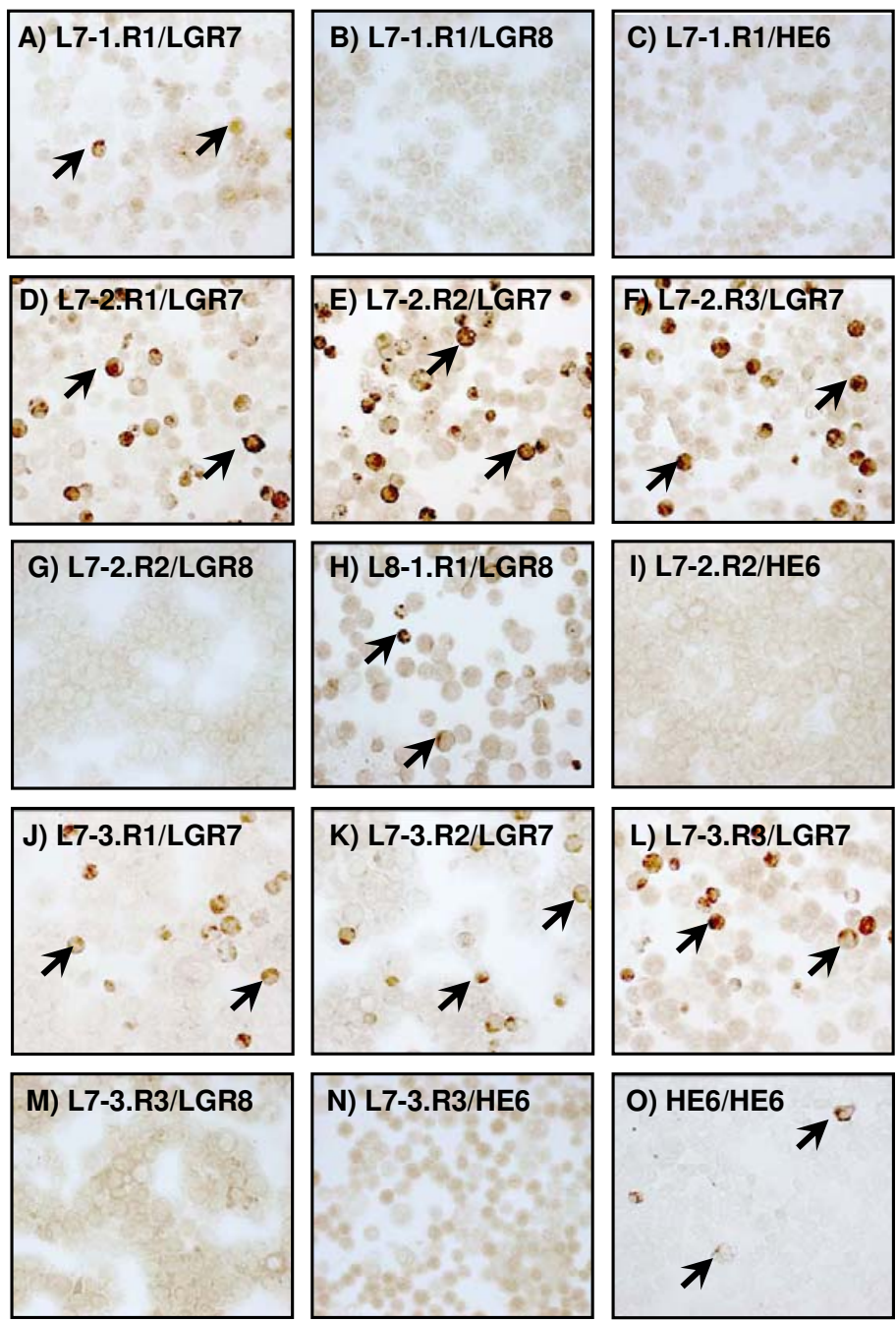
Figure 1
Immunohistochemical staining of cytospin preparations of HEC295T cells transfected or not with DNA constructs expressing either LGR7, LGR8 or the orphan G-protein coupled receptor, HE6. Each panel is annotated to indicate, from left to right, the epitope against which the antibody has been raised (e.g. L7-2), then the animal used for this epitope (e.g. R3), and finally the expression construct transiently transfected into the cells (e.g. LGR7, LGR8 or HE6). All antisera against epitopes L7-1 and L7-2 were diluted 1:4000: those against L7-3 were diluted 1:5000. Arrowheads point to positively immunostained cells. Negative controls are shown in panels B, C, G, H, M and N. Positive controls for the LGR8 and HE6 transfections are shown in panels H and O, respectively. Scale bar = 50 μm.
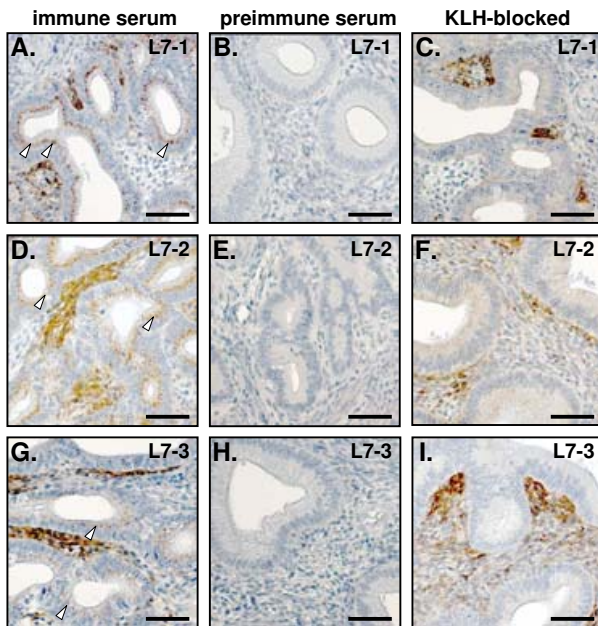
Figure 2
Immunostaining of sections of uterus (endometrium) from a single female cynomolgus macaque in the secretory phase of the estrous cycle. Specific LGR7 immunostaining is consistently observed in endometrial stromal cells for all three LGR7 epitopes (A, L7-1; D, L7-2; G, L7-3), which is not observed when applying the preimmune sera at the same concentration (B, E, H). Variable immunostaining is observed also in the glandular epithelial cells (A, D, G; arrowheads, which, however, is completely attenuated upon preincubating the diluted primary antisera with an excess of the hapten, KLH (C, F, I). Sera from only one animal was chosen for each epitope (L7-1, R1; L7-2, R2; L7-3, R3). A second monkey, also in the secretory phase, was also analysed in parallel with identical results (not shown). Scale bar = 50 μm.

Figure 3
Immunostaining of human uterus using antisera recognizing the L7-2 (R2) and L7-3 (R3) epitopes (A, B). Only a single patient in the mid-late secretory phase, is illustrated, since of other tissues examined from four other patients of the proliferative or early secretory phase, none showed consistent immunostaining (see text). This patient also exhibited small fibroid nodules (E, F) in the myometrium, which immunostained strongly and specifically for both LGR7 epitopes tested. The respective pre-immune sera from the same animals and at the same dilution as used for the primary antibodies, served as negative controls (C, D, G, H). Scale bar = 50 μm.

purified primary cells from endometrial stroma, epithelium and myometrium of human uterine samples obtained at hysterectomy from premenopausal patients with non-endocrine ailments. PCR primers were so chosen as to detect and distinguish both full-length LGR7 transcripts as well as the recently described [Hsu et al., 2002] splice variant. The results show that only a single LGR7 transcript is detectable in endometrial stromal cells, epithelial cells and myometrial cells (Fig. 4). Because these cells were cultured for several days, it is not possible to state whether the apparent differences in amounts are due to real differences in expression *in vivo*. However, it is clear from this experiment that the cycle-dependent effectors progesterone and relaxin have no effect *in vitro* on the le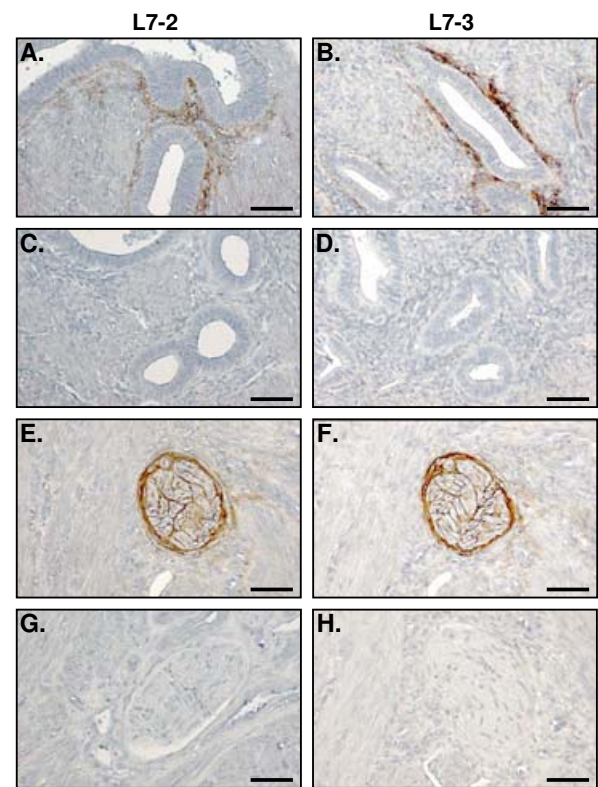vel of LGR7 transcript expression (Fig. 4A). Similar results were obtained using endometrial stromal cells under conditions known to promote decidualization *in vitro*, as evidenced by the induction of transcripts for prolactin (PRL) and IGF-binding protein 1 (IGF-BP1) (Fig. 4B). None of the effector combinations used, which included relaxin, progesterone and the specific phosphodiesterase 4 inhibitor, rolipram (Bartsch et al., 2004), had any influence on LGR7 transcript levels in these cells (Fig. 4B). Also direct application of the second messenger 8Br-cAMP failed to affect LGR7 transcript level. A more detailed analysis of the LGR7 transcripts expressed in the human endometrial stromal cells (Fig. 4C), using various

combinations of oligonucleotide primers covering most of the published transcript sequence, showed that even small transcript variants, such as could occur by alternative splicing of the several small exons in the region encoding the ectodomain, do not appear to be expressed in detectable quantities in these cells. Moreover, these PCR products were excised from the gels, sequenced and shown to be exactly as predicted from the published full-length LGR7 cDNA sequence [16].

Finally, uterine tissues from marmoset monkeys at known days within the estrous cycle, or within pregnancy were assessed using all three L7-1, L7-2 and L7-3 recognizing antisera. Figure 5 illustrates the results using the L7-1 antisera. The other sera gave identical images (not shown). Just as for the other primate species, specific immunoreactive signals were most evident in the stromal cells, particularly those close to glandular elements. Signal intensity was generally weak during the follicular phase (Fig. 5A,5B), though increased under the gonadotropin surge (Fig. 5C). Staining increased progressively through the luteal phase (Fig. 5D,5E,5F), and declined in intensity at the end of the luteal phase where no pregnancy had occurred (Fig. 5G). Besides the marked stromal cell staining, there is also clear epithelial immunostaining in some sections (e.g. Fig. 5A), which is not present in the negative controls (Fig. 5H,5I), and cannot be suppressed by preincubating the antisera with KLH (not shown).

Expression of LGR7 epitopes in normal and cancerous human breast tissues
Similar procedures were also carried out on normal lactating human breast tissue (Fig. 6), and on breast tissue containing tumors of various degrees of development and differentiation (Fig. 6). In the healthy breast tissue, and for most of the tumors examined, only the stromal compartment showed consistent and specific immunostaining of a similar character using the two most immunogenic LGR7 epitopes, L7-2 and L7-3. Staining is particularly marked in those regions of the stromal mesenchyme adjacent to glandular epithelia. In the healthy tissue the glandular and myoepithelium are consistently free of immunostaining. For the tumors a very similar picture is seen, also similar for the epitopes, L7-2 and L7-3 (Fig. 7). In occasional regions of the sections including breast tumor tissue, also some specific staining can be detected in epithelial-derived tumor cells (Fig. 6M,6N,6P,6R and 6S; arrowheads). Immunostaining is unaffected by pretreatment with the KLH hapten (Fig. 7).

Discussion
Antibodies represent one of the most sensitive instruments for the detection of receptor proteins *in situ* in tissue sections, and offer a high spatial resolution by comparison with radiolabelled ligand-binding or *in situ*

mRNA hybridization. The use of biotinylated ligands can also offer a high resolution, but may be accompanied by problems of non-specific binding. In a recent study where biotinylated relaxin was used to detect binding proteins and possibly receptors in the marmoset uterus, a method was employed which also indicated the electrophoretic mobility of the binding moieties [26]. Although some of the proteins detected might have represented receptors, other proteins of a diverse range of molecular weights were also observed, reinforcing the limited specificity of such methodology. We have made similar observations also for the human uterus (Y. Pohnke, unpublished observations). In the present study, polyclonal antibodies were raised against three quite different epitopes of the recently cloned LGR7 heptahelical domain relaxin receptor. For each epitope two or three rabbits had been immunized. Thus, as a first criterion of specificity, only those immunostaining features common to all three epitopes, and for more than one animal per epitope, can be considered as specific for the relaxin receptor. As a second criterion, all immunostaining was performed in parallel using as negative controls pre-immune sera from the same animals and at the same dilutions, under which conditions only the specific signals should not be detected. Thirdly, since all peptide epitopes were cross-linked to keyhole limpet hemocyanin (KLH) as a common hapten, it is possible that false positive immunostaining, meeting the above two criteria, could be obtained when antibodies recognizing the KLH cross-react with a mimetic epitope of some kind, though considerable experience of many scientists over the years suggests that this is a very unusual event for this hapten. To check for such source of error, antibodies were additionally pre-incubated with a large excess of pure KLH prior to applying them to tissue sections. The results showed that for the uterine sections of the cynomolgus macaque the staining in the epithelial cell layer may have been due to such false positive reactions. In contrast, the staining of the endometrial stroma remained quantitatively unaltered. In the human endometrium there was no such epithelial staining in the first place, reinforcing the view that, where this occurred in the macaque, this was indeed probably due to a false positive reaction. Finally, as positive controls, cytospin preparations were made of cell-lines having no endogenous relaxin receptors, as well as of similar cells transiently transfected with expression constructs encoding the LGR7 receptor, or alternatively the related receptors, LGR8 and HE6. All the LGR7 antibodies used showed clear positive staining only in those cells transfected by the LGR7 constructs, and neither in non-transfected cells nor in the cells expressing the other related receptors. In an independent study in the rat brain, the antibodies L7-2 and L7-3, whose immunogenic epitopes differ only slightly between humans and rats, indicate complete cellular identity between immunostaining, [33]P-
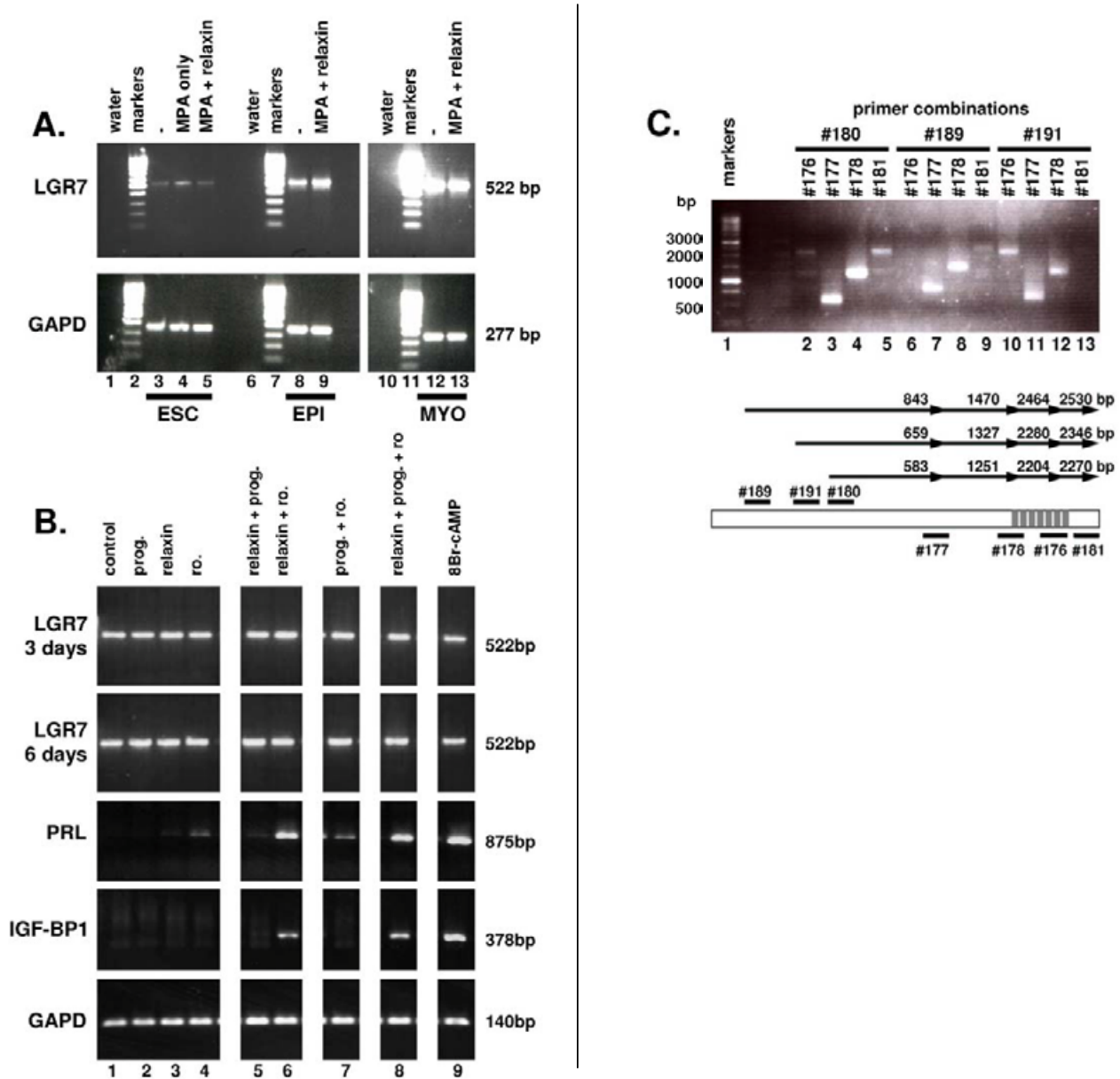
Figure 4

RT-PCR reactions for LGR7 gene transcripts in human uterine cells. **A**. Primary cultures of endometrium stromal cells (ESC), glandular epithelial cells (EPI) and myometrial smooth muscle cells (MYO) cultivated for 6 days and treated or not, as indicated with 2.5×10^7M medroxyprogesterone acetate (MPA) and/or 100 ng/ml porcine relaxin. The LGR7 PCR used primers #174 and #178, that for GAPD used #150 and #151. **B**. Primary cultures of endometrial stromal cells cultured for 3 or 6 days as indicated with progesterone (prog., 2.5×10^{-7}M), relaxin (100 ng/ml), rolipram (ro., 100 μM) and their combinations. RT-PCR products for prolactin (PRL), IGF-BP1 and GAPD mRNAs were performed in parallel on 6 day cultures only. All lanes are from the same gels run and photographed under identical conditions; the images have been cut to eliminate unwanted tracks only. PCR primers for LGR7 were as above, for GAPD #400 and #401 were used. **C**. RT-PCR reactions for RNA from a single preparation of ESC cells using different primer conditions (see lower panel) in order to determine the expression of possible LGR7 splice variants. The sizes of the DNA markers are indicated on the left of the upper panel. In the lower panel the sizes of the expected products, based on the published cDNA sequence, are indicated above the horizontal arrows. Primer #180 is positioned to include the start codon of the open reading frame, whereas #189 and #190 are both within the 5' untranslated region.
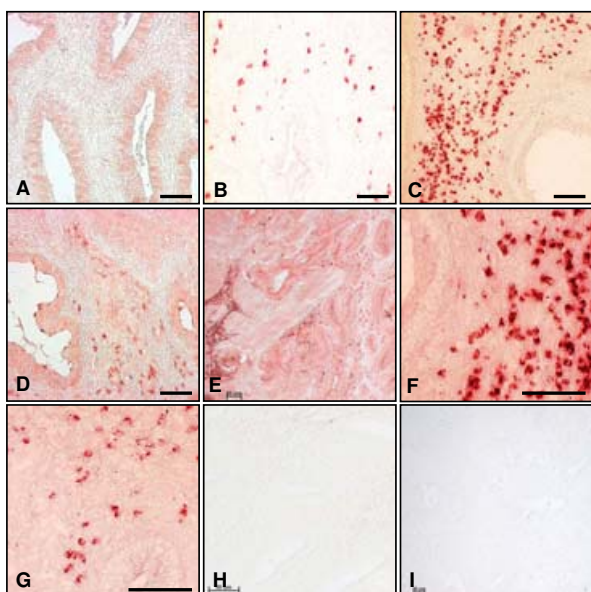
Figure 5
Immunostaining for LGR7 epitopes (L7-1) in the endometrium of the marmoset monkey. A, day 4 follicle phase. B, day 7 follicle phase. C, day 8 follicle phase, following administration of a single ovulatory dose of hCG. D, From another animal as in C, showing a larger overview. E, day 3/4 of the luteal phase, overview. F, As in E, detail. G, day 9 luteal phase. H, negative control using preimmune serum: day 8/9 of the follicle phase after application of hCG. I, negative control, day 3/4 of the luteal phase. Scale bars in all figures represent 50 μm.

Figure 6
Immunostaining for LGR7 epitopes of healthy human breast tissue from a lactating patient, undergoing mammotomy for incipient breast cancer. This tissue has been described elsewhere [42]. Antibodies against the epitopes L7-2 (panels A and C) and L7-3 (panels B and D) were used, and gave identical results (L7-2.R2 was used at 1:4000, and L7-3.R3 at 1:5000 dilution). The effect of preincubating the antibodies with an excess of KLH was also tested: A and B, without KLH; C and D, with KLH. The preimmune sera from the same animals used to generate the specific antisera were applied in E and F, at the same dilutions. Scale bar = 50 μm.

relaxin binding and *in situ* mRNA hybridization (T. Burazin, University of Melbourne, personal communication). Given these extensive controls, we can be confident that the immunostaining seen truly represents the relaxin receptor LGR7.

Within the uterus, marked specific immunostaining is seen in the endometrial stromal cells in human, macaque and marmoset uterus. The stroma is not uniformly stained but in patches within the *functionalis* but not in the *basalis*. Staining appears to be strongest in those stromal cells adjacent to, or circumscribing endometrial glands. The marmoset monkey offers an ideal model in which to study this expression, since tissues can be obtained from animals whose cycles are precisely synchronized by a luteolytic dose of prostaglandin-F2α [33], and hence at precise days of the cycle or pregnancy. Using such tissues, and in an independent laboratory, LGR7-immunostaining was observed essentially identical to that observed for the
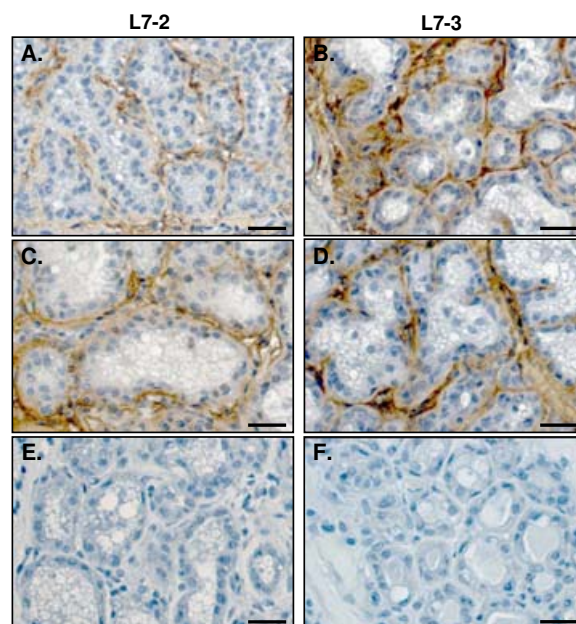
other species, with most being in the stromal cells close to endometrial glands. In the marmoset we can confirm the suggestion offered by the human tissues, that LGR7 immunoreactivity increases from the proliferative to the luteal pase of the cycle. It increases still further in early pregnancy, concomitant with a role in the decidualization process essential for implantation (not shown). The marmoset results were also fully coherent with the results of biotinylated-RLX binding [26], acting as a further control for the validity of the antibodies in these species.

The epithelial staining, observed consistently for some antibodies in the macaque, is attenuated by preincubaton of the antibodies with an excess of KLH and therefore probably represents unspecific false positive immunostaining. The weak but specific epithelial staining seen in the marmoset monkey appears to be truly specific and is
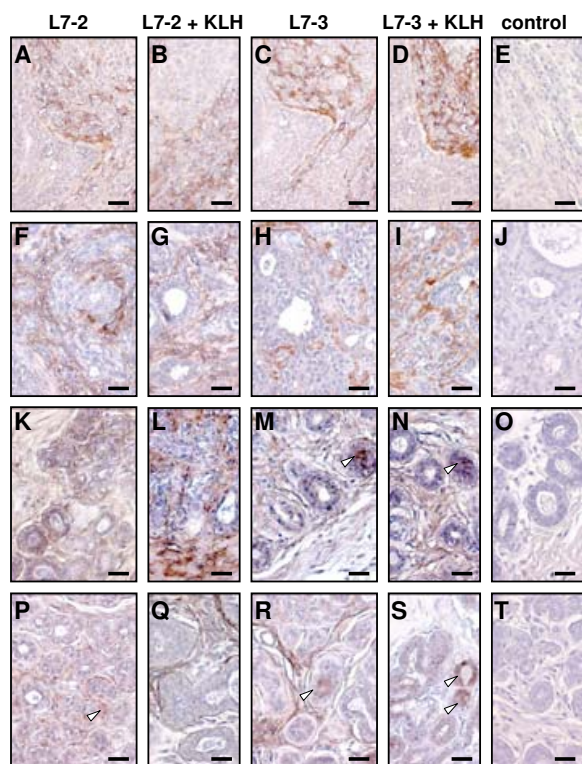
Figure 7
Immunohistochemistry of primary breast tumors from four different patients. A-E: ER- and PR-positive lobular breast tumor, grade 2. F-J: ER- and PR-positive ductal breast tumor, grade I. K-O: ductal breast cancer, also ER- and PR-positive, grade I. P-T: ductal tumor, ER- and PR-positive, grade 2. As in Fig. 5, all tumors are stained for both L7-2 (A, F, K.P) and L7-3 (C, H, M, R) epitopes in the absence of KLH preincubation, or in its presence (B, D, G, I, L, N, Q, S). Negative controls for both epitopes are also shown (E, T: preimmune serum for L7-2.R2; J, O: preimmune serum for L7-3.R3). Other controls were similar (not shown). Note that the weak, but specific immunostaining of the epithelial tumor cells (e.g. in M and R; arrowheads and elsewhere) was not suppressed by preincubation of the primary antisera with KLH. Scale bar = 50 μm.

not attenuated in KLH-suppression controls. Negligble immunostaining was observed in the myometrium in all three species. This may be due to true low level expression of the LGR7 receptor protein, though may represent a methodological problem; other receptor proteins in our hands have sometimes required a microwave antigen-retrieval protocol to be visualized in this tissue (MB, personal observations).

These observations of LGR7 immunoexpression in the uterus are very much in agreement with studies using purified human primary cell cultures. Relaxin has been shown to have an important effect in the induction of decidualization in endometrial stromal cells [1,37]. This effect is mediated by well characterized relaxin receptors on the cell surface, expressed at a concentration of ca. 1000 molecules per cell, and having a Kd of ≤ 1 nM [24]. Relaxin is also known to influence myometrial function by inhibiting spontaneous contractions, though these effects are most marked in rodents, and are considered not significant in the human [38]. Relaxin-binding is also reported for endometrial epithelial cells [25], and functional responses to relaxin have been reported also for these cells [9,39,40].

Using RT-PCR we can detect LGR7 mRNA in all three uterine cell types. Since these are cultured cells, however, these experiments cannot provide information about the levels of transcripts or protein in the intact tissues. Furthermore, the relative levels might be distorted by the proportion of cells expressing the LGR7 transcripts. The immunohistochemistry emphasizes that not all stromal cells express LGR7, but only some of them, and this probably in a cycle-dependent manner. Depending on the ratio of expressing to non-expressing cells in the primary cultures, the observed mRNA concentrations in the extracted RNA samples could vary greatly. What is significant, however, is that known uterine effectors, like progesterone and relaxin, have no effect on the LGR7 gene expression in these cultured cells. This would imply that any cycle-dependent variation *in vivo* requires a more complex collection of factors and/or paracrine interactions. Transcripts for the shorter splice variant do not appear to be expressed in the cultured cells to any significant degree. Thus, although potentially this transcript could give rise to a free ectodomain, which has experimentally been shown to bind relaxin [16], there is still no evidence that such splice variants are expressed or translated *in vivo*, or that any such free ectodomain is exported from the cells.

Of particular interest, was the observation in one patient of fibroid nodules in the myometrium, which were clearly enveloped by positively immunostaining connective tissue cells. While this is possibly part of the normal encapsulation response towards such fibroids, it provokes the question of what role relaxin and its receptor could be playing in the etiology and pathology of this debilitating ailment.

In the normal and pathological human breast, LGR7 immunoreactivity is prominent in the stromal connective tissue between the glandular lobules. Epithelial cells of the healthy breast are unstained. Such epithelial staining does appear to occur sporadically in breast tumor tissue,

which would be in agreement with the findings that the breast cancer cell-line MCF7, believed to be of epithelial origin, is also responsive towards relaxin [12]. While the tumor tissue is mostly unstained, in some tumors weak and localized staining can be observed. Several studies have looked at a role for relaxin in human breast function, particularly in the context of cancer [11,13]. It is reported that breast cancer cell lines can respond to relaxin by a change in growth parameters, probably involving local NO production [41]. More interestingly, it has recently been shown that women with metastatic breast cancer have significantly higher serum relaxin concentrations than those with non-progressing cancer [14]. Furthermore, breast cancer cells treated with relaxin show a significant increase in invasive potential, using a classic *in vitro* invasion assay [15]. These studies thus emphasize the importance of relaxin on connective tissue remodelling in the breast particularly in relation to cancer progression and metastasis formation, and support well our observations using the new LGR7 antibodies that the relaxin receptor is prominently expressed on the connective tissue cells responsible for creating a capsule around a growing tumor, as well as on some tumor cells of epithelial origin in breast cancer patients. The similarity here to the situation for the uterine fibroids is striking.

The present article describes a new panel of polyclonal antibodies generated against the human LGR7 receptor. Using a combination of immunochemical, molecular biological and immunohistochemical techniques we here provide a first validation of these antibodies and demonstrate their value in studying the physiology and pathology of the human breast and uterus.

Authors' Contributions

RI was responsible for all conceptual work, project coordination and management. MB performed the antibody validation and immunohistochemisty on all human and macaque tissues. YP, RT and OB, carried out the molecular biology studies on the cytospin validation of antibodies using transfected expression constructs. Additionally, RT and OB were responsible for cell culture and RT-PCR studies. AMB and KML provided human uterine and breast tumor tissues and contributed to the histological characterization of these sections. AE was responsible for all work on the marmoset.

Acknowledgements

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