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Abstract

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Growth and characterization of different human rhinovirus C types in three-dimensional human airway epithelia reconstituted in vitro

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New molecular diagnostic tools have recently allowed the discovery of human rhinovirus species C (HRV-C) that may be overrepresented in children with lower respiratory tract complications. Unlike HRV-A and HRV-B, HRV-C cannot be propagated in conventional immortalized cell lines and their biological properties have been difficult to study. Recent studies have described the successful amplification of HRV-C15, HRV-C11, and HRV-C41 in sinus mucosal organ cultures and in fully differentiated human airway epithelial cells. Consistent with these studies, we report that a panel of clinical HRV-C specimens including HRV-C2, HRV-C7, HRV-C12, HRV-C15, and HRV-C29 types were all capable of mediating productive infection in reconstituted 3D human primary upper airway epithelial tissues and that the virions enter and exit preferentially through the apical surface. Similar to HRV-A and HRV-B, our data support the acid sensitivity of HRV-C. We observed also that the optimum temperature requirement during HRV-C growth may be type-dependent.

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Introduction

Human rhinoviruses (HRVs) are classified into three species (HRV-A, -B and -C) based on capsid sequence relatedness (Laine et al., 2006; Ledford et al., 2004; Savolainen et al., 2002). By contrast to their closest relatives, the human enteroviruses (HEVs), which can cause disseminated disease and affect multiple organs including the central nervous system, HRVs infect primarily the upper respiratory tract. This restricted tropism is in part attributed to temperature constraints needed for optimal growth and acid sensitivity. HRVs and HEVs present different optimal growth temperature in vitro. HEVs grow better at 37 °C, whereas most HRVs grow best at 33 °C, the temperature of the upper airways. Furthermore, compared to HEVs, HRVs are sensitive to acidic pH and do not survive the gastrointestinal acidic environment. Although acid sensitivity seems related to capsid stability (Skern et al., 1991), the genotypic determinants of temperature adaptation are not known. We highlighted a higher GC content among HEVs than among HRVs (Tapparel et al., 2007). Whether a causative relationship exists between this feature and temperature adaptation remains to be demonstrated. However, temperature and acid sensitivity may not be true for all HRVs. Some HRV types were shown to grow equally or even better at 37 °C (Papadopoulos et al., 1999) and an increasing number of reports have found HRV nucleic acids in stools (Harvala et al., 2012; Lau et al., 2012), while Honkanen et al. could even isolate the virus in stool samples of young children (Honkanen et al., 2012). These findings suggest that at least some HRV types are able to survive both the higher temperature and acidic environment found in the gastrointestinal tract.

HRV-C was identified in 2006 (Arden et al., 2006; Gerna et al., 2007; Kiang et al., 2007; Kistler et al., 2007; Lamson et al., 2006; Lau et al., 2007; Lee et al., 2007; McErlean et al., 2008; McErlean et al., 2007; Remwick et al., 2007) and revealed to have been circulating for hundreds of years in humans (Briese et al., 2008; Huang et al., 2009; Wisdom et al., 2009). Epidemiological studies have shown that HRV-As are the most prevalent rhinoviruses in human infections (45% to 65% of cases), closely followed by HRV-Cs (30% to 50%) and HRV-Bs (2% to 13%) (Arakawa et al., 2012; Harvala et al., 2012; Ouyang et al., 2012; Tapparel et al., 2011; Wisdom et al., 2009). Reports suggest that HRV-Cs may be overrepresented in children with pneumonia or acute wheezing and exacerbation of asthma, suggesting an association between HRV-C infection and lower respiratory tract complications (De Angelis et al., 2010; Lau et al., 2007; Miller et al., 2009; Tapparel et al., 2009b; Wisdom et al., 2009). Disseminated HRV-C infections...
have also been documented (Broberg et al., 2011; Fuji et al., 2011; Tapparel et al., 2011). However, strong evidence is lacking to definitely claim that HRV-Cs might be more virulent or more adapted to the lower airway environment (Iwane et al., 2011; Lee et al., 2012; Tapparel et al., 2011).

To better appreciate HRV-C biological properties and pathogenesis, functional culture systems are needed since this virus does not grow in standard cell lines. Three studies have recently described the successful amplification of HRV-C15 clinical specimen in sinus mucosal organ cultures (Bochkov et al., 2011), HRV-C15 and HRV-C11 generated from infectious clones in fully differentiated human airways' epithelial cells (nasal or bronchial epithelial) (Hao et al., 2012), and HRV-C15 and HRV-C41 clinical specimens in differentiated sinus epithelial cells (Ashraf et al., 2012). The effects of antibodies directed against HRV major (intercellular adhesion molecule-1 [ICAM1]) or minor group (low density lipoprotein receptor [LDLR]) receptors on HRV-C replication were assessed in these culture models and confirm that HRV-Cs use a different receptor. Furthermore, Ashraf et al. showed that two HRV-C types (HRV-C15 and HRV-C41), although similar to HRV-A and -B viruses in relation to acid sensitivity, displayed similar growth efficiency at 34 °C and 37 °C, suggesting that this HRV species may be more adapted to the lower airway temperature.

In this study, we used commercially available, 3D human upper airway epithelia reconstituted in vitro (MucilAir™, Epithelix, Geneva, Switzerland) to extend these observations using HRV-C infected clinical specimens. We found that this primary air–liquid interface (ALI) culture system supports successful growth of eight out of eight tested clinical strains, representing five different HRV-C types (HRV-C2, -C15, -C29, -C12 and -C7). We demonstrate also that HRV-Cs enter and exit preferentially at the apical side of this ALI culture system. Finally, we confirm the acid sensitivity of HRV-Cs and show that temperature sensitivity may be type-dependent.

Results

Different HRV-C types grow in air–liquid interface (ALI) Human airway epithelia reconstituted in vitro (MucilAir™)

Among upper respiratory tract samples collected from patients of the University of Geneva Hospitals, screened positive for HRV infection and typed based on VP4/VP2 sequencing, seven specimens infected with HRV-C viruses and one (WW74-1202-U) with HRV-A41 were used for this study. A phylogenetic tree based on the VP4/VP2 sequence of these HRV-C clinical strains and on corresponding sequences of HRV-C reference types (http://www.picornastudypgroup.com/types/enterovirus/hrv-c.htm) shows that these selected strains were distributed among five distantly related HRV genotypes (HRV-C2, -C7, -C12, -C15 and -C29 [Fig. 1]). The calculated VP4/VP2 nucleotide sequence homology for each of them with the closest reference type ranges from 92.1% to 97% (Table 1). Each of these HRV-C clinical samples, HRV-A41 clinical sample, and HRV-A16 stock was inoculated on ALI human airway epithelia reconstituted in vitro (named thereafter MucilAir™ for simplicity), and the HRV RNA load was quantified at the apical surface at different times post-inoculation by real-time RT-PCR (Schibler et al., 2012b; Tapparel et al., 2009a). The RNA load present in the HRV inoculum is indicated for each clinical specimen (Fig. 2). The RNA level observed at the apical surface after the 6 h incubation period (presumably before virion production) and after repeated PBS washes was considered as a reference background level for each specimen analyzed. Eight out of eight clinical strains tested, as well as HRV-A16, display replication in MucilAir™ with a maximum RNA load observed between 24 h and 72 h (Fig. 2). The increase in cell-associated virus tested for the HRV-C29 specimen also revealed significant replication with a maximum yield observed for an inoculum of 10^5.4 HRV copies (Fig. 3). Of note, this inoculum load represents RNA copies and may be an overestimation of the number of fully competent virions present in the clinical specimen. In addition, HRV-A16, HRV-C7, and HRV-C29 virions collected from the apical surface or from total tissue lysates were subsequently passed and re-amplified in MucilAir™ (data not shown). The presence of replicating virus was further confirmed by both immunofluorescence and in situ hybridization on total MucilAir™ or on paraffin-embedded tissue sections (Fig. 4). The localization of infected cells confirms that viral replication is restricted to cells located at the apical surface of the MucilAir™ for both HRV-A and HRV-C (Fig. 4).

HRV-Cs enter and exit predominantly at the apical surface of the human airway epithelium

To test the preferred HRV entry site in the polarized epithelium, HRV-C7 and HRV-A16, were inoculated at the apical (tissue/air interface) or at the basal (basal/liquid interface) side of the MucilAir™, respectively, and virus production was quantified by real-time RT-PCR from both sides at different times post-infection. Virus inoculation at the basal surface did not give rise to any viral RNA production, neither from the apical nor the basal surface (data not shown). By contrast, after apical inoculation of four different HRV-C types, HRV RNA could be recovered from both the apical and basal sides, but viral RNA was detected with higher and more sustained loads in the apical compared to the basal surface of the epithelium (Fig. 5). Of note, the higher load observed at the apical surface is not linked to RNA leftover from apical inoculation since inoculation by total immersion of the tissue in diluted clinical specimen gave rise to the same observation (data not shown). Furthermore, no HRV RNA could be found at 48 h after apical inoculation with the same amount of acid-treated (Fig.5) or UV-inactivated virus (data not shown).

HRV-Cs are sensitive to acidic pH, whereas temperature sensitivity may be type-dependent

Clinical strains corresponding to HRV-C29 and HRV-A41, as well as HRV-A16, were inoculated on MucilAir™ after pretreatment with acidic (pH 4) or neutral (pH 7.2) solution. In parallel, a well characterized HEV-A71 (HEV-A71 [EU414331]) was treated similarly before Vero cell infection. Neither HRV-C29 and HRV-A41 nor HRV-A16 were able to grow after acidic pretreatment, whereas HEV-A71 grew efficiently independent of any acidic pretreatment (Fig. 6).

The growths of HRV-C2, -C7, -C12 and -C29, as well as HRV-A41 clinical strains, were compared at 34 °C and 37 °C. Fig. 7 shows that HRV-C2 and -C12 strains present almost equivalent replication levels at both temperatures, similar to the HRV-A41 strain and the HEV-A71 [EU414331] control tested on Vero cells (data not shown). By contrast, HRV-C7 and HRV-C29 strains show a significant growth advantage at 34 °C 24 h post inoculation while this growth advantage gets smaller at later time points. Of note, the calculated GC content of each of these different viruses based on the GenBank complete genome sequence of the nearest serotype (if available) (Fig. 7) did not show a correlation with optimal growth temperature in this experiment.

Discussion

The commercially available, reconstituted, MucilAir™ used in this study allowed to investigate the biological properties of HRV-C
viruses. It sustained replication for HRV-A16 and for eight clinical strains corresponding to five different HRV-C types and an HRV-A41. These MucilAir present most of the morphological and functional features of native tissues, such as the presence of basal, mucus, and ciliated cells; secretion of cytokines, chemokines and various proteases; active cilia beating and mucociliary clearance; ion channel activity and electrophysiology [http://www.epithelix.com/content/view/6/5/lang,en]. In addition, these tissues are cultured in ALI, which allows the study of the viral life cycle in the context of a polarized tissue. Finally, the quality control performed before tissue delivery and the reconstitution of human airway epithelia from a pool of nasal polyp cells originating from different donors reduce considerably the experimental variability observed with organ cultures (Bochkov et al., 2011).

Fig. 1. Phylogenetic tree of the different HRV-Cs sequenced from the clinical samples used in this study. The VP4/VP2 nucleotide sequence (405 nt from the ORF start codon) of HRV-C-positive clinical samples (in bold) and representatives of each of the 51 HRV-C reference types whose sequence is available in GenBank were included in the phylogenetic analysis with enterovirus 68 (EV68, GenBank ID: AY426531) as the outgroup. Only bootstrap values > 50 are indicated. Genbank ID of each Genbank available sequence is indicated in brackets next to the sequence name.

Table 1
Identity of the different HRV-C clinical strains and nearest reference types. The percent of nucleotide homology between each clinical strain and the nearest reference types are indicated. For Genbank ID, see Fig. 1.
When setting up the optimal conditions for MucilAir™ infection, we observed no replication when the virus was added at the basal side of the epithelium, whereas replication was optimal after inoculation at the apical surface. This suggests that HRVs enter at the apical side of the upper respiratory epithelium. Immunoﬂuorescence with an antibody directed against double-stranded RNA, or in situ hybridization with peptide nucleic acid-based hybridization oligoprobe speciﬁc for HRVs and HEVs then showed that both HRV-Cs and HRV-As present a replication restricted to the upper layers of the epithelium, thus conﬁrming previous observations (Arruda et al., 1995; Bochkov et al., 2011; de Arruda et al., 1991; Hao et al., 2012). Finally, we showed that HRV-Cs exit preferentially from the apical side of the epithelium. Thus, we postulate that these viruses infect the upper layers of the respiratory epithelium, replicate in these superﬁcial layers, and ﬁnally exit at the apical surface. This would explain in part why HRVs are restricted to the respiratory tract and do not disseminate in general. It will be interesting to use this polarized tissue culture system to ﬁnd out whether viruses that can disseminate after entry via the respiratory route present the ability to exit preferentially from the basal side of this reconstituted human airway epithelium.

We analyzed the acid sensitivity of HRV-C29 and reproduced the observation made for HRV-C15 (Ashraf et al., 2012), i.e., HRV-Cs appear acid-sensitive, similar to HRV-As and HRV- Bs. We then compared the optimal growth temperature of strains corresponding to four different HRV-C types used in this study and found that temperature sensitivity at early time points differed among the tested clinical strains. Indeed, HRV-C7 and HRV-C29 grew better at 34°C than at 37°C 24 h post-inoculation with a three-log replicative advantage for HRV-C7. Surprisingly, this advantage was reduced at later time points. Whether this is linked to viral adaptation after several rounds of replication remains an open question. The other tested HRV-C types, as well as an HRV-A clinical strain (HRV-A41), showed a limited or no replicative advantage at 34°C, suggesting that optimal growth temperature may be type-dependent for HRV-Cs as observed previously for other HRVs. The study by Ashraf et al. showed equivalent replication for both HRV-C15 and HRV-C41 at 34°C and 37°C.
U), as well as acid-inactivated HRV-C29 (see Fig. 6), were inoculated at the apical surface of MucilAir.

Resistant to higher temperatures, we did not coxsackie A1 and HEV-D70 (Tapparel et al., 2012). Additional with low GC content (43%) can cause gastroenteritis, such as discovered HEV-C104, -C109, -C117 and -C118. Inversely, viruses enteroviruses present high GC content (48%), such as the recently associated with respiratory infections, other respiratory frequently associated with respiratory infections, other respiratory enteroviruses present high GC content (48%), such as the recently discovered HEV-C104, -C117 and -C118. Inversely, viruses with low GC content (43%) can cause gastroenteritis, such as coxsackie A1 and HEV-D70 (Tapparel et al., 2012). Additional investigations are required to establish a strict link between GC content and given phenotypes.

If some HRV-C types are more resistant to higher body temperatures, they may have an improved propensity to infect the lower airways. Genotyping of HRV-C strains found in lower respiratory tract complications could help to answer this question. Similarly, HRVs isolated from stools should support higher body temperatures, in addition to being acidic-resistant. Thus, it will be of interest to genotype and sequence the complete genome of HRVs isolated from stool samples (Harvala et al., 2012; Honkanen et al., 2012; Lau et al., 2012).

In conclusion, we consider this commercially available, standardized, reconstituted human upper airway epithelium ALI culture system as a useful tool to model the respiratory epithelium and to study infections not only caused by HRVs, but also by other respiratory pathogens. Our observations help to understand some of the clinical phenotypes observed during natural HRV-C infections.

Materials and methods

Viruses, clinical specimens and sequences

HRV-A16 and HEV-A71 [EU414331] viral stocks were prepared from infectious clones in Hela Ohio and Vero cells as previously described (Cordey et al., 2012; Schibler et al., 2012a). Nasopharyngeal swabs of patients enrolled in clinical studies approved by the University of Geneva Hospitals ethics committee and screened positive for HRV were used for the study. The VP4/VP2 sequences of these clinical HRV strains were obtained by semi-nested PCR amplification and sequencing with the HRV forward (F484-5'CG-GCCCCCTGAATGYGGCTAA3' and F587sn-5'TCATTGTGGTGTCCGTGTTTCT3') and reverse primers (R1126-5'ATCHGHARYTCCAMCACCA3') as previously described (Linsuwanon et al., 2009; Tapparel et al., 2011) and are available in Genbank (accession numbers KC841446 to KC841451 and HM347252 and HM347267).

Human airway epithelium reconstituted in vitro (MucilAir™)

Nasal polyp epithelial cells were obtained from patients undergoing surgical nasal polypectomy. All experimental procedures were explained in full, and all subjects provided written informed consent. The study was conducted according to the Declaration of Helsinki on biomedical research (Hong Kong amendment, 1989), and the research protocol was approved by the local ethics committee.

To reconstitute the human airway epithelium in vitro, low passage (P0 or P1) primary cells were seeded in 6.5 mm Transwell-COL inserts (0.4 µm pore size, Costar 3470, Corning Inc.). Once the cells were attached to the semipermeable membrane, the culture medium was removed from the upper chamber of the inserts and the cells were then cultured at the air–liquid interface (ALI culture). After approximately 45 days of culture, the epithelium became fully differentiated, both morphologically and functionally, i.e., the epithelium showed a pseudo-stratified architecture with the three main types of epithelial cells (ciliated, mucus, and basal cells). The epithelium showed active cilia-beating and mucociliary clearance, as well as ion channel activities. Furthermore, once differentiated, it remains at a homeostasis state for more than one year. This ALI culture is known commercially as MucilAir™. Some epithelia were prepared with a mixture of cells originating from different donors (14 in total), so-called MucilAir™ “pool”, which were reconstituted in the same way as MucilAir™ and with the same characteristics. The MucilAir™ pool is available in large quantities and for several years. The differentiated epithelium contains approximately 400,000 cells. All inserts were tested (Ashraf et al., 2012). Of note, these HRV-C types are phylogenetically related (Fig. 1) and may thus belong to a group of HRV-Cs resistant to higher temperatures. We did not find a strict correlation between temperature sensitivity and GC content among the HRV types tested. This is also the case among other viruses of the Enterovirus genus. Although most HRV-As and HRV-Bs present a GC content below 41%, similar to HEV-D68, an enterovirus frequently associated with respiratory infections, other respiratory enteroviruses present high GC content (48%), such as the recently discovered HEV-C104, -C109, -C117 and -C118. Inversely, viruses with low GC content (43%) can cause gastroenteritis, such as coxsackie A1 and HEV-D70 (Tapparel et al., 2012). Additional investigations are required to establish a strict link between GC content and given phenotypes.
negative for HIV-1, mycoplasma, hepatitis B, hepatitis C, bacteria, yeast, and fungi.

Infection

HRV clinical specimens were either undiluted or diluted in MucilAir™ ready-to-use culture medium (Epithelix). Before inoculation, MucilAir™ was washed three times with Ca²⁺-Mg²⁺-free phosphate-buffered saline (PBS−). Tissues were then either totally immersed in the viral suspension, or 100 μl or 500 μl of viral suspension were applied on the apical or basal face, respectively, of the epithelium. At 4 or 6 h post-incubation at 34 °C or 37 °C, tissues were rinsed three times with PBS− and cultures were continued in the liquid air interface with 500 μl of fresh culture medium. For HRV-A16, HRV-A41, HRV-C15, and HRV-C29 types, successive passages were performed as follows: apical washes were collected at 48 h post-infection and 1/5 volume was used to infect new MucilAir™ tissues.

For the acid sensitivity assay, 20 μl of clinical samples containing HRV-C29, as well as HRV-A41, HRV-A16 and HEV-A71 [EU414331] viral stocks, were diluted in 1 volume (20 μl) of 0.1 M citrate buffer pH 4.0 or 0.1 M of phosphate buffer pH 7.2. Samples were then incubated for 1 h at 37 °C before neutralization by the addition of 2 volumes (40 μl) of 0.1 M phosphate buffer pH 7.2 and 6 volumes (120 μl) of culture medium with a final 10-fold dilution of the clinical specimen, and inoculated onto tissue or vero cell cultures.

RNA load quantification

At different times post-inoculation, an apical wash was collected by applying 200 μl of culture media to the apical surface of the MucilAir™ and incubating for 20 min at 34 °C or 37 °C. For collection from the basal face, the 500 μl culture media was collected and replaced with fresh media. To quantify cell-associated viruses, total tissue lysates were collected 4 h or 24 h post-incubation at 34 °C.

For virus RNA load quantification, dilutions applied during sample processing (from extraction to real-time PCR) were taken into account in the final calculation. RNA quantification was performed by one-step real-time RT-PCR as previously described (Schibler et al., 2012b; Tapparel et al., 2009a). HRV RNA was measured with the Panenterhino/Ge/08 assay using HEV-14 10-fold diluted RNA transcript as reference standard, while HEV RNA was quantified with the Entero/Ge/08 assay using HEV-71 (EU414331) 10-fold diluted RNA transcript as reference standard. Canine distemper virus (CDV) was spiked as a control in each extraction as previously described. For cell-associated virus quantification, viral amplicon Ct values were normalized according to the endogenous RNase P gene (TaqMan RNase P Control Reagents, ref 4316844, Applied Biosystems).

Immunofluorescence

MucilAir™ infected for 48 h or 10 days by different clinical HRV-C specimens or HRV-A41 and non-infected epithelia were rinsed three times with PBS− and fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. Tissues were then washed twice with PBS−, incubated in ammonium chloride (NH₄Cl) solution (50 mM in PBS, pH 8.0) for 15 min at room temperature, and then permeabilized with 0.05% Triton X-100 in PBS− for a further 15 min. After three washes with PBS− containing 1% newborn calf serum (PBS-NCS), tissues were incubated for 1 h at room temperature with a primary mouse antibody (mAbJ2) (Engscicons, Cat 10010200) targeting double-stranded RNA and validated for wide HRV detection (Jurgeit et al., 2010). After five washes, tissues were incubated with Alexa 594-conjugated secondary anti-mouse IgG as described (Sobo et al., 2011). Tissues were then washed five times with PBS-NCS, incubated in 4, 6-diamino-2-phenylindole (DAPI), washed five times with PBS-NCS, and mounted onto glass slides. Alternatively, immune staining was performed on paraffin-embedded MucilAir™ tissue sections. In this case, paraffin was removed by warming the slices at 95 °C in 100 mM EDTA before staining.

Fig. 7. HRV-A and HRV-Cs clinical specimens were inoculated on MucilAir™ grown at 34 °C or 37 °C and viral load was quantified from the apical surface at different times post-infection. The GC content of each of these different viruses was calculated based on the GenBank complete genome sequence of the nearest type (Genbank EF077280 for HRV-C2, DQ879532 for HRV-C7, GU219984 for HRV-C12, and DQ473491 for HRV-A41) when available and is indicated in brackets. The error bar indicates the standard deviation calculated on biological replicates [n = 3 for HRV-C2 (VD11-1202-U), HRV-C12 (GM09-0909-U), HRV-C29 (KG11-1202-U) and HRV-A41 (WW74-1202-U9) and n = 2 for HRV-C7 (WA23-1203-U)]. P < 0.0001; P = 0.056. NA: not available.
Images were acquired in multiple sections (0.3 µm/section, 1 µm/section for paraffin-embedded slices and total tissues, respectively) from the apical to the basal pole using a Zeiss LSM 510 Meta confocal laser scanning microscope, and were processed by Imaris bitplane software as described (Sobo et al., 2012). All images presented are three-dimensional (3D) projections from the multisecton scannings.

**In situ hybridization**

Infectious MucilAir™ was labeled by *in situ* hybridization with the PNA ISH detection kit (DakoCytomation, Baar, Switzerland) and a peptide nucleic acid-based hybridization oligoprobe (Panenterhino/Ge/08 PNA probe) designed to detect all HRV and HEV species members as previously described (Tapparel et al., 2009a). In brief, tissue sections were rinsed twice with PBS— fixed with 4% PFA for 30 min at room temperature. After a quick wash with PBS— immersed in 1 ml PBS— for 10 min at room temperature. They were then permeabilized with proteinase K (provided with the kit) diluted (1:100) in Tris-buffered saline (TBS) for 30 min at room temperature in a moist chamber. Tissues were rinsed with PBS— briefly fixed again for 5 min with 4% PFA at room temperature, and washed for 5 min in PBS—. Hybridization was carried out according to the guidelines of the kit provider. The probe was added to the tissues and incubated at 55 °C for 1.5 h followed by four successive washing steps (the first two steps for 10 min and the last ones for 20 min each) using the stringent washing solution (Dako Permanent Red detection kit) at 60 °C. Hybridization was revealed with the fluorescent red sub- strate (Dako Permanent Red detection kit). Tissues were incubated in DAPI for 5 min at RT and mounted onto glass slides for confocal microscope analysis.

**Statistical analysis**

To evaluate the statistical significance of differences in RNA quantification obtained between two groups, *P* values were determined using the *t*-test (Fig. 7) or the Mann–Whitney test (Fig. 5). *P* < 0.05 was considered significant.

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