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# Spatiotemporal expression of Rhomboid domain containing 2 (Rhbdd2) during rat development

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ABSTRACT

Over the last few years rhomboid genes have gained interest because of its association with cancer and neurodegenerative diseases. In previous studies, we demonstrated that human RHBDD2 is over-expressed in the advanced stages of breast and colorectal cancers, suggesting a favorable role in cell proliferation. So far little is known about the expression of RHBDD2 in other tissues and other species, and because of similarities between cancer and embryonic cells, this study focused on the evaluation of Rhbdd2 expression in embryonic and adult rat tissues. By IHC and RT-PCR, Rhbdd2 was identified in early stages of most tissues analyzed, with high expression in brain, spinal cord, kidney and embryonic skin. In adult tissues, the expression remained elevated while salivary glands became positive. Furthermore, Rhbdd2 showed a high expression in the most proliferative stages of the rat mammary gland. Indeed, similar findings were observed in the mouse mammary epithelial cell line HC11, in which Rhbdd2 resides in the Golgi apparatus, and at different stages of mouse mammary gland development. Therefore, Rhbdd2 would be implicated in embryonic and adult tissue proliferation.



## 1. Introduction

The Rhomboid family is a heterogeneous group of membrane proteins, with and without protease activity, involved in various functions such as cell signaling, development, stem cell differentiation, apoptosis or stress response to endoplasmic reticulum (ER) (Freeman, 2008; Bergbold and Lemberg, 2013). Several members have been associated to neurodegenerative diseases and cancer (McQuibban et al., 2003; Urban and Freeman, 2003; Cipolat et al., 2006; Zou et al., 2009; McQuibban and Bulman, 2011; Adrain et al., 2011; Fleig et al., 2012). Despite the diversity of functions, which are restricted to a small group of members of the family, the biological role of many others remains unknown.

In previous studies, we determined that the expression of the gene RHBDD2, a family member without protease activity, is increased in the advanced stages of breast and colorectal cancer, suggesting that high RHBDD2 expression might have a favorable effect on tumor cell behavior (Abba et al., 2009; Lacunza et al., 2012). In a subsequent study, we showed that silencing RHBDD2 in breast tumor cells affects the expression of genes associated with the regulatory program of the ER stress known as the unfolded protein response (UPR), with a significant decrease in cell migration, proliferation and anchorage-independent growth processes. These results led us to speculate that RHBDD2 expression could be associated with an adaptive response of the cells to thrive under stressful conditions (Lacunza et al., 2014). Furthermore, in other study conducted in mice, the authors analyzed Rhbdd2 expression in retina, and determined that Rhbdd2 is expressed from embryonic stages to adulthood, and its levels show age-dependent changes, suggesting that Rhbdd2 fulfill an important role in the development and normal function of the retina (Ahmedi et al., 2013).

Based on all previous results and considering that normal development of an embryo requires a fine tuning between cell proliferation, migration, differentiation, survival and apoptosis, we hypothesize that Rhbdd2 expression might be an early event during development.

In this respect, Rhbdd2 expression was analyzed at mRNA and protein levels by RT-PCR and IHC, respectively, in several tissues of rat embryonic stages and adults. A cell line and tissues of mouse mammary gland were also evaluated for Rhbdd2 expression and sub-cellular localization.

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## 2. Materials and methods

### 2.1. Animals and samples

Briefly, a total of 48 animals of *Rattus norvegicus* (WKAH/Hok) were included in this study: 40 embryos and fetuses of pregnant days (D) 16, 17, 18, 19 and 20, and 8 adults of 10–12 weeks of age; from each pregnant day 8 individuals were collected. Copulation was determined by the presence of a vaginal plug; the middle of the *artificial night* was designed as day 0 of pregnancy (Theiler, 1989).

The following organs were studied: esophagus, stomach, small intestine, large intestine, liver, pancreas, salivary glands, trachea, lung, kidney, adrenal gland, skin, encephalon and spinal cord. From adults, mammary glands in their different cycles of proliferation were also obtained (Juvenile, Juv; Pubertal, Pub; Virgin Adult, Vir; Pregnant, Pre; Lactating, Lac; Post-lactating, Pos, and Senile Involution, Sen) (Masso-Welch et al., 2000). Also, samples of mouse mammary gland in two stages: virgin adult and lactating were analyzed in this study. The procedure was carried out in accordance with the *Guide for the Care and Use of Laboratory* Animals published by the Institute for Laboratory Animal Research (Washington DC, USA, 2011) Protocol was approved by CICUAL (Institutional Committee on Care and Use of Experimental Animals) from the Faculty of Veterinary Sciences, University of La Plata. The animals were sacrificed in chambers containing $CO_2$; immediately, animals were dissected and the embryos and fetuses were removed. Sterile surgical material was used and worked on ice.

### 2.2. Tissue processing

Embryos and fetuses were obtained and processed according to previous studies (Lacunza et al., 2010). They were obtained by laparotomy and hysterectomy of the pregnant females. The number of breeding in each gestation ranged from 4 to 12.

Whole embryos and fetuses as well as adult organs, were separated into groups, each of which was processed according to the methodology employed. For immunohistochemistry analysis (IHC), they were washed with 0.01 M phosphate buffer saline pH 7.4 (PBS) and fixed in 10% formaldehyde solution for 3 h. Samples were finally paraffin-embedded and sectioned with a thickness of 6 μm. To analyze mRNA expression, fresh tissue was placed into *RNAlater*® (Invitrogen Corp., CA, USA) to preserve the integrity of the sample. TRIzol reagent was used for RNA isolation (Invitrogen, San Francisco, CA, USA).

### 2.3. Immunohistochemical analysis (IHC)

Immunohistochemistry was performed according to standard protocols as reported in a previous study (Lacunza et al., 2010). Briefly, dewaxed sections were treated with 10 mM sodium citrate buffer at 100 °C for 5 min for antigen retrieval and were placed in methanol with 0.3% $H_2O_2$ to block endogenous peroxidase activity; after three washes in PBS, sections were blocked for non-specific binding with normal horse serum diluted 1:10 in 1% bovine serum albumin in PBS, (BSA)/PBS. Samples were then incubated overnight with anti-human RHBDD2 polyclonal antibody (TA306891, Origene, USA) (1 μg/ml, dilution 1:150), which cross-reacts with rat and mouse, at 4 °C, whereas negative controls were incubated with PBS under the same conditions. Briefly, immunodetection was performed with the DakoCytomation LSAB + System-HRP (Dako, Denmark). Finally, sections were counterstained with hematoxylin (Sigma), dehydrated and coverslipped with mounting media. Samples were evaluated under light microscope and the reaction was considered positive when more than 5% of the cells were stained. Staining intensity was scored in a semiquantitative manner and was graded as negative (−), low (+), moderate (++) and strong (+++).

### 2.4. HC11 cell culture

In order to evaluate Rhbdd2 mRNA expression in an in vitro model of mammary gland differentiation we employed the HC11 mouse cell line. When these cells are treated with lactogenic hormones they synthesize the milk protein beta casein, an established marker of mammary epithelial cell differentiation. To optimally respond to lactogenic hormones and thus achieve the differentiated stage, HC11 cells must grow to confluence, a stage defined as competent, which is preceded by a proliferative stage. HC11 cells were grown in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (FCS) and 5 mg/ml insulin (Sigma). Confluent HC11 cells were maintained in RPMI with 2% FCS and 5 mg/ml insulin for 3 days after which 5 mg/ml PRL (Sigma) was added.

### 2.5. Rhbdd2 co-localization

Based on the expression pattern frequently observed with Rhbbd2 immunoreaction in this and previous studies, we infer that Rhbdd2 could sub-localize in the endomembrane system, mainly ER and/or Golgi apparatus. To evaluate this, HC11 cells were harvested, spread on slides and fixed with cold acetone for 10 min; washed in PBS and blocked with horse serum for 15 min. The rabbit polyclonal antibody anti-RHBDD2 (TA306891, Origene, USA) and the mouse antibodies, markers for the ER and the Golgi apparatus, anti-calnexin (Abcam, USA) and anti-Tn (MA1-23598, Affinity BioReagents, USA), respectively, were used. Cells were incubated with the respective antibodies for 12 h and then washed in PBS. For the primary mouse monoclonal antibodies, anti IgG mouse with biotin was employed as secondary antibody, incubated for 30 min, followed by incubation with fluorescein streptavidin (1: 1000) mixed with propidium iodide (1/100). Anti IgG rabbit with Cy3 (Jackson ImmunoResearch, USA), was used for RHBDD2 antibody (1/400). Slides were observed in an inverted fluorescence confocal microscope (LSM410, Zeiss). Images were captured and analyzed with the ImageJ software and JACOP application that allows the calculation of Manders' Overlap Coefficient (MOC).

### 2.6. RT-PCR analysis of Rhbdd2 mRNA

With the aim of obtaining a correlation between RNA and protein expression, total RNA was isolated from the embryonic organs pancreas, stomach, kidney, skin and small intestine, adulthood rat and mouse mammary glands of different proliferation cycles and HC11 cell line, using TRI Reagent® (MCR Inc., USA). Following DNAse I digestion, cDNAs were synthesized using SuperScript™ First-strand Synthesis System (Invitrogen, USA) and measured in a NanoDrop Spectrophotometer® 2000. For mRNA expression analysis, 500 ng of total cDNA for each sample was employed. The following primers were designed and used: Rhbdd2 (Rattus norvegicus), forward 5′-CTGAGCGAGTAGCCCTGAAG-3′, reverse 5′-CATCTGGGTGACAGGGTGAC-3′; Rhbdd2 (Mus musculus) forward 5′-TGGTGTTCGGTGTGGTGGT-3′, reverse 5′-GCCATAGGACAGGCCAATCA-3′, Csn2 (Mus musculus), forward 5′-ACTCTCAAATCCCCAGCCTTG-3′, reverse 5′-TCGTTCATCGTGGGAAGGAAG-3′; RNA18S, forward 5′-GTAACCCGTTGAACCCCATT-3′, reverse 5′-GCGATGATGGCTAACCTACC-3′. Thermal profile for Rhbdd2 was programmed as follows: an initial denaturation step of 3 min at 95 °C followed by 40 cycles of 40 s at 95 °C, 30 s at 64 °C and 30 s at 72 °C and a final extension at 72 °C for 3 min. Detection of the amplified fragments was made by electrophoresis onto 2% agarose

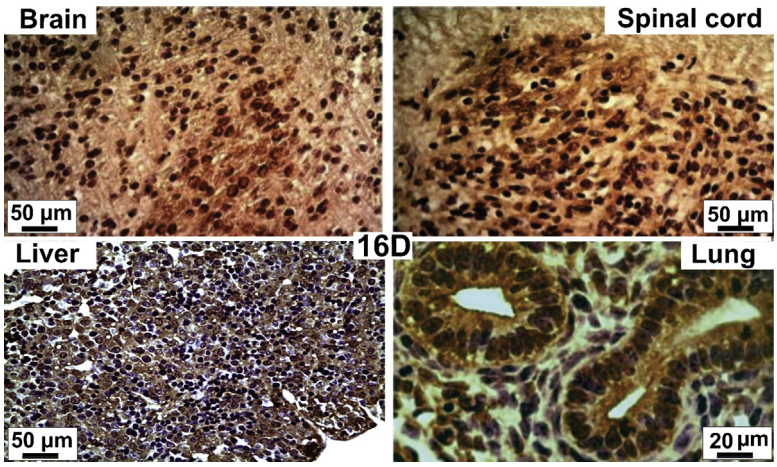


**Fig. 1.** Rhbdd2 protein expression evidenced in brain, lung, liver and spinal cord of rat embryos at 16 days of gestation (40×). High Rhbbd2 expression is observed in all cases with a predominantly cytoplasmic pattern.

gels and GelGreen staining (Biotium, USA). Experiments were performed in triplicate and RNA 18S was used as reference.

## 3. Results

### 3.1. Rhbdd2 expression during development

In most tissues analyzed the expression levels of Rhbdd2 protein gradually increased from day 16 (D16) to D20 and remained high in adults (Table 1).

### 3.2. Embryos and fetuses

Immunohistochemistry analysis revealed that the earliest expression of Rhbdd2 protein was at D16 in the encephalon, spinal cord, lung, liver, kidney, adrenal glands and skin, and remained expressed during development of these organs. A cytoplasmic pattern of reaction was predominant, showing in some cases an apical and a nuclear pattern. The encephalon and spinal cord were positive with a low to moderate intensity and a cytoplasmic pattern, some cells also showed nuclear reaction (Fig. 1); in the liver, Rhbdd2 expression was restricted to the cells of the hepatic cords (Fig. 1) whereas a moderate apical reaction was observed in the primordial lung buds (Fig. 1); the nephric tubules of the developing kidney also showed an apical pattern of reaction, as well as in the adrenal secretory cells while the embryonic epidermis reacted with a cytoplasmic and low staining.

At D17 stage the intensity of reaction of kidney, adrenal gland, embryonic epidermis, encephalon and spinal cord increased in comparison with D16. Fig. 2 shows the high expression of spinal cord and lung while esophagus is not reactive.

At D18 stage Rhbdd2 expression appeared in pancreas, stomach and small and large intestines. In the stomach, the reaction was restricted to the developing glands, with a moderate intensity and an apical pattern; small and large intestines showed a similar pattern of reaction at developing Lieberkühn glands. In stomach and pancreatic cells a weak cytoplasmic reaction was observed. Also, nephric tubules of the developing kidney and cortical and medullar cells of the adrenal glands showed a stronger reaction with respect to previous stages (Fig. 2).

At D19 of gestation Rhbdd2 protein expression increased in pancreatic acini with a cytoplasmic pattern but a low intensity of reaction (Fig. 2).

At 20 days of development the intensity of Rhbdd2 reaction increased in the skin, the hepatocytes and gastric and intestinal glands (Fig. 2).

Salivary glands, esophagus and trachea were negative in all stages.

Rhbdd2 expression in embryonic tissues was validated at the RNA level, showing in most cases a correlation with protein results. A slight difference was observed for pancreas, small intestine and stomach, in which Rhbdd2 RNA was found in previous stages to protein detection (Fig. 3).

**Table 1**
Immunohistochemical results of Rhbbd2 protein in different tissues from rat embryos, fetuses and adults. Intensity of reaction: +, low; ++ moderate; +++ intense; –, no reaction.

| Organs | Embryos and Fetuses Gestational days | | | | | Adults |
|---|---|---|---|---|---|---|
| | 16 | 17 | 18 | 19 | 20 | 10–12 weeks |
| Esophagus | – | – | – | – | – | – |
| Stomach | – | – | ++ | ++ | +++ | ++ |
| Small intestine | – | – | ++ | ++ | +++ | +++ |
| Large intestine | – | – | ++ | ++ | +++ | +++ |
| Pancreas | – | – | + | ++ | ++ | ++ |
| Liver | + | + | ++ | ++ | +++ | ++ |
| Salivary gland | – | – | – | – | – | ++ |
| Trachea | – | – | – | – | – | – |
| Lung | ++ | ++ | ++ | +++ | +++ | +++ |
| Kidney | + | ++ | +++ | +++ | +++ | +++ |
| Adrenal gland | + | ++ | +++ | +++ | +++ | +++ |
| Skin | + | ++ | ++ | +++ | +++ | ++ |
| Encephalon | ++ | ++ | +++ | +++ | +++ | +++ |
| Spinal cord | ++ | ++ | +++ | +++ | +++ | +++ |

### 3.3. Adults

The same tissues were also analyzed for Rhbdd2 expression in adult rats. Rhbdd2 was expressed in 100% (8/8) of the samples of stomach, adrenal gland, liver, small intestine and kidney, 75% (6/8) of the samples of brain, spinal cord, lung and skin and 62, 5% (5/8) of pancreatic samples. Compared with embryonic stages, Rhbdd2 was detected in salivary gland, being positive 37, 5% (3/8) of the samples analyzed. In most cases the reaction was cytoplasmic (Fig. 4). Esophagus and trachea did not show reaction.

In the stomach Rhbdd2 expression was restricted to the glandular region (Fig. 4). The small and large intestines staining was observed at the apical part of the columnar epithelial cells and Lieberkühn glands (Fig. 4) with a variable intensity, from moderate to strong. In hepatocytes Rhbdd2 expression was observed with a cytoplasmic pattern and a strong intensity. In the kidney the

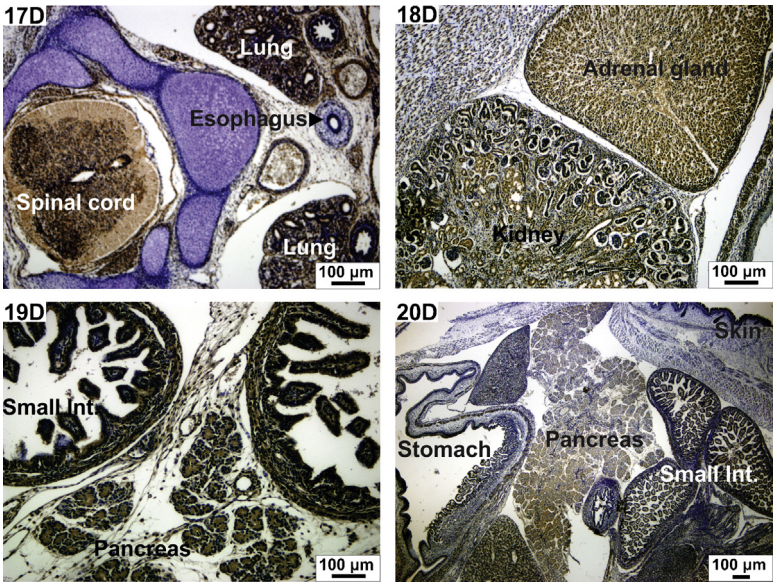


**Fig. 2.** Rhbbd2 protein expression in different tissues from rat fetuses. A positive reaction is observed in spinal cord and lung (17D) (10×), adrenal glands and kidney (18D) (10×), small intestine and pancreas (19D) (10×), stomach and skin (20D) (4×), whereas no reaction was observed in esophagus (17D).

reaction was limited to the epithelial cells of the tubules, and in the lung, to the epithelial cells of bronchi and bronchioles. Adrenal cortex and medulla showed a cytoplasmic reaction. Serous and mucous acini of salivary glands (Fig. 4) and pancreatic acini showed a moderate cytoplasmic expression. In the epidermis, the positive reaction was observed in the sebaceous glands. Brain specimens showed high expression of Rhbdd2 with a cytoplasmic pattern, and a nuclear pattern in some cells.

### 3.4. Mammary gland

We evaluated Rhbdd2 expression in adult rat mammary gland at different proliferative stages (Fig. 5a and b). The analysis was extended to mouse mammary gland (Fig. 5c and d). IHC analysis showed Rhbdd2 expression at the pregnant and lactating stages with a strong intensity in all pregnant samples and a moderate to strong intensity in lactating samples, whereas a weak reaction was observed in the virgin adult stage (Fig. 5a). The pattern of reaction was mainly cytoplasmic, with an apical localization in some cells. Juvenile, pubertal, post-lactating and senile involution stages were all negative. To determine if protein expression correlated with RNA detection, RT-PCR analysis was performed. Results indicated that Rhbdd2 appears in the juvenile stage, its level increase to the pregnant stage and decline sharply in the post-lactating stage (Fig. 5b).

Furthermore, we employed the HC11 mouse mammary cell line to evaluate Rhbdd2 expression at mRNA and protein levels by RT-PCR and WB, respectively. We also found a high expression of RHBDD2 mRNA at the proliferative stage of the HC11 cell line with a dramatic decrease at the competent and differentiated stages, being the expression higher in the latter (Fig. 5c). Similar results were obtained in tissues from mouse mammary gland of virgin adult and lactating stages (Fig. 5d).

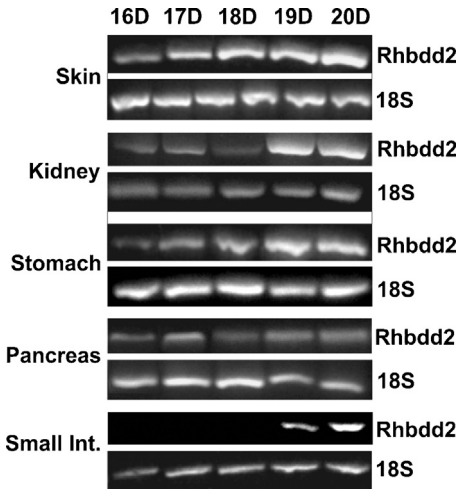


**Fig. 3.** Rhbdd2 mRNA profile in various tissues from rat embryos and fetuses at different gestational ages.

### 3.5. Rhbdd2 localization in HC11 cells

HC11 was also used to evaluate Rhbdd2 sub-cellular localization. Cells were double labeled with anti-RHBDD2 and alternatively with an antibody marker of the ER (anti-calnexin) or an antibody marker of the Golgi apparatus (anti-Tn). When the images of both combinations were merged, we found that Rhbdd2 co-localizes with Tn (MOC = 0.97) (Fig. 5e). Moreover, overlapping with calnexin was not significant (data not shown).

## 4. Discussion

In this study we evaluated the pattern of expression of Rhbdd2 protein in various tissues obtained from different stages of rat development, which included embryos of 16–20 days of gestation and adults. This is the first study on evaluating Rhbdd2 expression at different tissues during rat development.

In previous studies we determined that RHBDD2 gene is over-expressed in the advanced stages of breast and colorectal cancers, suggesting a relevant role in tumor progression (Abba et al., 2009; Lacunza et al., 2012). Also, we observed a positive association between RHBDD2 expression and a low grade of differentiation (high rate of proliferation) in breast cancer tissues (Abba et al., 2009; Canzoneri et al., 2014). In a later study, microarray analysis of breast cancer cells with silenced RHBDD2 gene, indicated a functional enrichment of bioprocesses associated with protein folding, protein transport and ER stress, among others. Furthermore, when

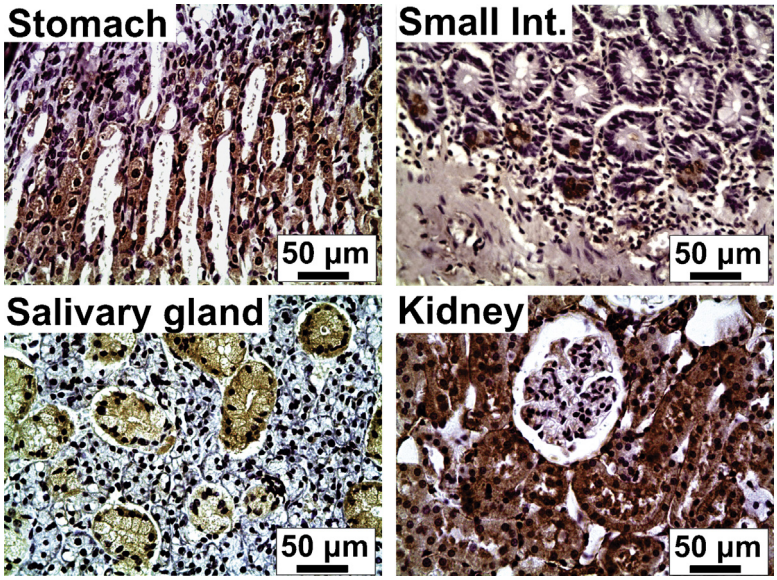


**Fig. 4.** Rhbbd2 protein expression detected in adult rat tissues (40×). A moderate to strong reaction is observed in gastric and intestinal glands, acinar cells from salivary glands and epithelial cells from renal tubules.

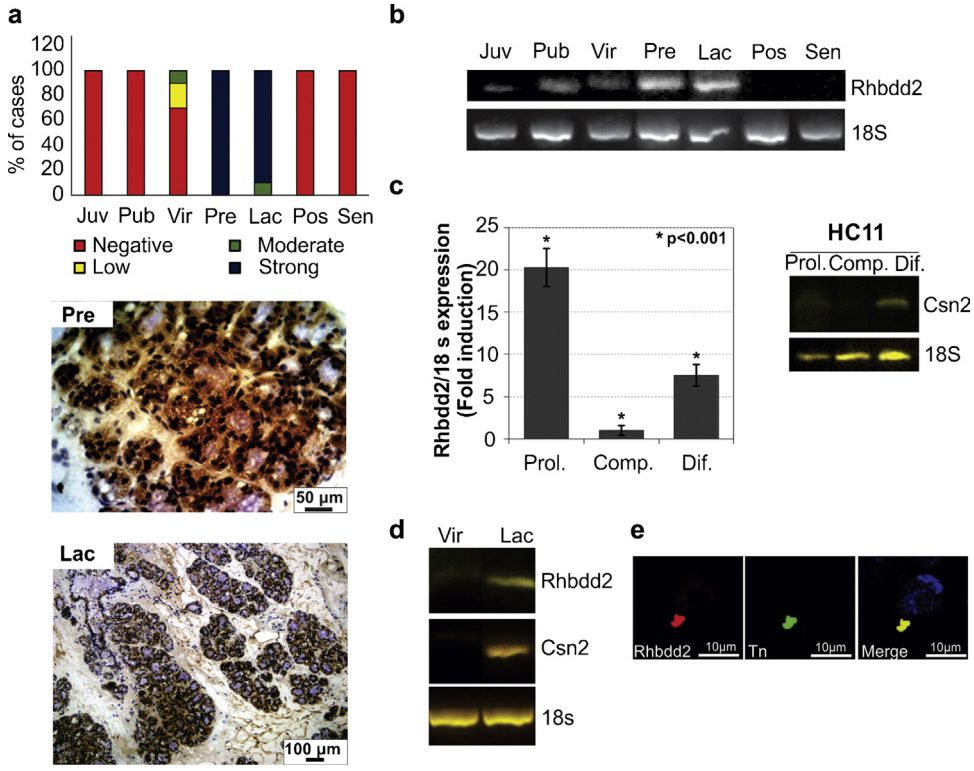


**Fig. 5.** Rhbdd2 expression in mouse mammary gland. (a) Bar chart representing the percentage of cases for Rhbdd2 protein expression in different cycles of mammary gland proliferation according to the intensity of reaction (above). Rhbbd2 protein expression in tissue sections of rat mammary gland at pregnant (Pre) and lactating (Lac) stages (40× and 10× respectively, below). An intense reaction with a cytoplasmic pattern is observed. (b) Rhhbdd2 mRNA expression in the mammary gland stages Juvenile (Juv), Pubertal (Pub), Virgin Adult (Vir), Pregnant Adult (Pre), Lactating Adult (Lac), Post-lactating Involution (Pos) and Senile Involution (Sen). An increase or Rhbdd2 expression is observed at the most proliferative stages. (c) Rhbbd2 mRNA expression in the mouse mammary gland cell line HC11. These cells acquire three stages: proliferative, competent and differentiated. Rhbdd2 mRNA levels are significantly higher in the proliferative stage, decreases abruptly in the competent cells and slightly increases in differentiated cells, stage defined by the expression of milk proteins such as beta-casein. (d) Rhbdd2 mRNA expression in lactating (Lac) and virgin (Vir) mouse mammary gland. Csn expression was used as an indicator of the lactating phenotype, whereas 18 s was used as a normalizer. (e) Representative confocal microscopy images of intracellular colocalization of Rhbdd2 protein (red) and the Golgi marker Tn antigen (green) in the HC11 cells (100×). The merged images showed a highly significant overlapping (yellow; MOC = 0.97). Nucleus can be observed lightly stained blue in the merged figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

these cells were subjected to stress conditions with DTT agent, a significant deregulation of the UPR genes expression as well as a decrease in cell migration and cell proliferation processes was observed (Lacunza et al., 2014). Although RHBDD2 function is still elusive, all these data strongly suggest that RHBDD2 gene would be associated with the biosynthetic pathway and its abrogation in cancer cells would impact in general processes such as cell proliferation or migration.

It is well known that cancer and embryonic cells share many characteristics; they are undifferentiated and proliferative and, while malignant cells migrate to distant sites to metastasize, embryonic cells migrate to form different tissues and organs (Nieto, 2013). Therefore, the high expression of RHBDD2 gene in the advanced stages of cancer – which are more undifferentiated than early stages (Perou et al., 2000) – and the decrease in cell proliferation and migration with RHBDD2 abrogation, raised the interest to investigate the expression of Rhbdd2 in healthy embryonic tissues.

By IHC, it was possible to identify Rhbdd2 from stage 16 in brain, spinal cord, adrenal glands, lung, kidney and liver. In most cases the pattern of reaction was cytoplasmic. In several tissues, however, a nuclear reaction was also observed.

At day 18 we detected Rhbdd2 in pancreas, stomach and small and large intestines, whereas the intensity of the reaction of the other reactive tissues increased respect to the previous stages. Interestingly, gland morphogenesis in the intestines and pancreas begins between the stages 17 and 18 (Hisaoka et al., 1993; Familari et al., 1998; Velcich et al., 2002).

No significant differences were observed in later embryological stages except for a slight increase in the intensity of the reaction and a higher proportion of positive nuclei in the brain and spinal cord in comparison to the earlier stages.

In a recent study of Rhbdd22 expression in rat retina, Ahmedi et al. (2013) suggested that Rhbdd2 has glycine zipper motifs in its sequence that are important to localize the protein in the Golgi membranes. Although the pattern of reaction observed in our study would be coincident, in some tissues, Rhbdd2 was detected in all the cytoplasm and at nuclear level, which indicates more than Golgi localization. In fact, in silico analysis of RHBDD2 protein sequence indicated enrichment in the TGN (Trans Golgi Network) endosomal pathway motifs, suggesting that RHBDD2 could also be taking part of the endosomal or vesicles membranes, which are usually distributed in the whole cytoplasm. Thus, by confocal microscopy and image analysis we decided to evaluate RHBDD2 sub-cellular localization in the HC11 cell line. We employed markers of the ER and Golgi apparatus and we observed predominately a perinuclear pattern, in which RHBDD2 co-localized with the Golgi marker Tn (GalNac), which is in agreement with the previous finding of Ahmedi et al. (2013), suggesting that Rhbdd2 mainly resides in the Golgi membranes. However, we cannot rule out the possibility that Rhbdd2 also be expressed in the membrane of secretory vesicles, which could explain the cytoplasmic pattern observed beyond the perinuclear region in many of the analyzed tissues. Specific markers of membrane vesicles would be needed to corroborate this.

Regarding the nuclear expression, it was more evident in brain and spinal cord tissues. It is probably that an alternative isoform of Rhbdd2 is being detected in these tissues. In this sense, in a previous study we identified two isoforms of RHBDD2 in breast cancer cells (Abba et al., 2009). Ahmedi et al. also found two Rhbdd2 isoforms in mouse retina and, in addition, they identified a third isoform in human retinal samples. NCBI Gene bank revision indicates two isoforms for human RHBDD2, an *isoform a* with 364 aa and a shorter *isoform b* lacking the first 142 aa. There is still no information about the existence of Rhbdd2 rat isoforms. Further studies are needed to elucidate whether human isoforms are always expressed together and in the same sub-cellular localization, or if they are under different tissue specific regulatory mechanisms.

IHC results were subsequently validated at mRNA level by RT-PCR. Overall, Rhbdd2 mRNA data are in agreement with our Rhbdd2 protein expression analysis. Curiously, Rhbdd2 mRNA of pancreas and digestive mucosa was identified in previous stages to IHC findings, which would suggest that protein levels at these stages are very low to be detected by IHC.

In summary, Rhbdd2 expression in the analyzed tissues could be divided in two groups, one of high expression, including brain, spinal cord, adrenal gland, kidney, lung and skin, and the other one of low to moderate expression, with stomach, small and large intestines, pancreas and liver.

Rhbdd2 expression was also evaluated in different stages of mammary gland development. Interestingly, Rhbdd2 appeared in the juvenile stage and gradually increased to the pregnant and lactating stages, being slightly higher in the pregnant and decaying abruptly to the following stages; the senile involution and post-lactating. Similar results were observed in mouse mammary gland, with a high expression at lactating stage and a weak reaction in the virgin adult stage. It has been established that during mammary gland morphogenesis, stress signaling is activated, involving genes of the UPR pathway (Avivar-Valderas et al., 2014). The high expression of Rhbdd2 in the most proliferative stages of mammary gland morphogenesis and the ulterior declination, coupled with our previous study of RHBDD2 expression under stressful conditions might suggest that Rhbdd2 expression favors cells to overcome stressful conditions, such as those found in the proliferative and migratory cycles of the mammary gland, during embryonic development or in tumor growth. Moreover, we employed the in vitro model of mouse mammary gland cell differentiation HC11 to analyze Rhbdd2 mRNA. These cells have been widely used as a suitable model to study mammary epithelial cell proliferation, signal transduction and differentiation in vitro. Mammary epithelial cells requires the interaction with extracellular matrix in order for the cells to respond to lactogenic hormones (Akhtar et al., 2009), and HC11 produces their own extracellular matrix proteins (Chammas et al., 1994). The cells in the undifferentiated and proliferative stage will become competent to respond to the lactogenic hormone prolactine and will differentiate to produce the milk protein β-casein among others (Ball et al., 1988). The production of β-casein indicates that HC11 cells likely contain an alveolar progenitor cell population that differentiates to form a milk protein secretory cell. Rhbbd2 showed a high expression in the proliferative stage, a dramatic decrease in the competent stage and a slight increase in the differentiated stage. This result is coincident with the observed in rat mammary gland and in our previous studies in human breast cancer, indicating that Rhbdd2 is more expressed in proliferative cells.

All tissues that showed reaction at embryo stage remained positive during adulthood; an exception was the salivary gland which showed Rhbdd2 expression only in adult tissues, which would be coincident with its functionality that begins after birth.

We conclude that Rhbdd2 shows a ubiquitous expression in early development, being high in glandular tissues or/and tissues with strong secretory activity, in which endomembrane system is highly developed. In this regard, we determined that in HC11 cells RHBDD2 resides in the Golgi apparatus. Finally, Rhbdd2 presence is important not only in developing tissues but also during proliferation of adult tissues.

## 5. Conflict of interest

The authors declare that they have no conflict of interest.

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