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Distribution of Calcitonin Gene-Related Peptide and Vascular Endothelial Growth Factor in the Mouse Lung at the Embryonic and Postnatal Stages

Distribución del Péptido Relacionado con el Gen de la Calcitonina y el Factor de Crecimiento Endotelial Vascular en el Pulmón de Ratón en las Etapas Embrionaria y Posnatal

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SUMMARY: Neuropeptide calcitonin gene-related peptide (CGRP) is a neurotransmitter related to vasculogenesis during organ development. The vascular endothelial growth factor A (VEGF-A) is also required for vascular patterning during lung morphogenesis. CGRP is primarily found in organs and initially appears in pulmonary neuroendocrine cells during the early embryonic stage of lung development. However, the relationship between CGRP and VEGF-A during lung formation remains unclear. This study investigates CGRP and VEGF-A mRNA expressions in the embryonic, pseudoglandular, canalicular, saccular, and alveolar stages of lung development from embryonic day 12.5 (E12.5) to postnatal day 5 (P5) through quantitative real-time polymerase chain reaction (qRT-PCR) and *in situ* hybridization. Further, we analyzed the expression of CGRP via immunohistochemistry. The VEGF-A mRNA was mainly scattered across the whole lung body from E12.5. CGRP was found to be expressed in a few epithelial cells of the canalicular and the respiratory bronchiole of the lung from E12.5 to P5. An antisense probe for CGRP mRNA was strongly detected in the lung from E14.5 to E17.5. Endogenous CGRP may regulate the development of the embryonic alveoli from E14.5 to E17.5 in a temporal manner.

KEY WORDS: Neurotransmitter; Calcitonin gene-related peptide; Vasculogenesis, Embryonic, postnatal; In situ hybridization.

INTRODUCTION

In mice, during the early stages, lung morphogenesis is a complex process that begins with the appearance of lung buds on embryonic day 9.5 (E9.5) (Bolte *et al*., 2018). There are five stages of lung development spanning from the embryonic to the postnatal period: the embryonic, pseudoglandular, canalicular, saccular, and alveolar stages (Schittny, 2017). The lung comprises blood vessels, stroma, and epithelial cells; all these change during lung development (Marszalek *et al*., 1999). All major alterations in respiratory function are attributable to changes in the chest wall compliance with lung tissues; such changes occur during the embryonic to postnatal periods of development. Moreover, restriction protocol of movement stress in pregnant mice induces the pulmonary histological changes in the fetal lung during development (Henríquez *et al*., 2018). The wood smoke pollution during pregnancy in a rat model was effect to the terminal bronchioles in the fetal rat lung (Salinas *et al*., 2020). Calcitonin gene-related peptide (CGRP) such as stress marker was expressed in the lung during E11–E15 (Wuenschell *et al*., 1996). CGRP provides an integrated mechanism response according to the changing circumstances (Hewitt & Lloyd, 2021). CGRP has various functions in terms of vascular and inflammatory regulations in the umbilical vein endothelial cells, lung smooth muscle, dendritic cells, bone, macrophages and lymphocytes (Nagashima *et al*., 2019). CGRP may relate to the respiratory bronchioles and bronchioles associated with the features of lung diseases of pulmonary fibrosis (Lv *et al*., 2020). As the bronchial tree extends, these cells give rise to populations of ciliated and pulmonary neuroendocrine cells in the

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developing lung at E18.5 (Morimoto *et al*., 2012). However, there are few reports about CGRP expression in cells during lung formation. The vascular endothelial growth factor (VEGF-A) is associated with the development of pulmonary fibrosis, with a paradigm of regulating tissue repair (Barratt *et al*., 2017). VEGF-A is also an important marker during lung development (Woik & Kroll, 2015). CGRP and VEGF co-play a role in human epithelial cells of the skin (Kay *et al*., 2014) and rib formation (Sawada *et al*., 2022). Despite CGRP being a potent vasodilator, information about the interaction between CGRP and VEGF-A is unknown. Therefore, the present study investigated the relationship between CGRP and VEGF-A mRNA expressions in lung development using *in situ* hybridization and quantitative real-time polymerase chain reaction (qRT-PCR). The results of this study provide useful information about lung development.

MATERIAL AND METHOD

Sample preparation. All laboratory animals were derived from Jcl:ICR mice, obtained from the Animal Resource Laboratory (CLEA Japan, Inc. Tokyo, Japan), and prepared by the Animal Testing Centre based on medical, biological, and drug development studies in accordance with the animal welfare regulations of CLEA Japan, Inc. Because mouse thorax development is more prominent in males than in females, we selected male mice for our research. The mice lung samples were collected on E12.5, E14.5, E17.5, P0, and P5. The samples from each stage (n = 4, total 20) were frozen at -80 °C and were subsequently prepared for q-RT-PCR. Other fixed tissues samples from each stage (n = 4, total 20) were used for *in situ* hybridization methods.

Determination of Sex. All samples were verified as male origin by RT-PCR with the primer pair 5'-TGG TCT GGA CCC AAA CGC TGT CCA CA-'3/5'- GGC AGC AGC CAT CAC ATA ATC CAG ATG-'3 and boiled embryonic tissues were used as templates. This protocol was modified by Sawada methods (Sawada *et al*., 2022).

qRT-PCR analysis of mRNA. The lung tissues were isolated under a stereomicroscope (LEICA MZ16; Leica Microsystems GmbH, Wetzlar, Germany). Total RNA (20–50 μg) was isolated from the lung samples of E12.5, E14.5, E17.5, P0, and P5 mice using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Other protocol following modified by Sawada methods (Sawada *et al*., 2022). qRT-PCR analysis was performed using the LightCycler® 96 Instrument (Roche Molecular Systems, Inc., Pleasanton, CA, USA) according to the manufacturer's instructions. Each 50-μl reaction volume contained 100 ng of cDNA, 900 nM forward primer, 900 nM reverse primer, 250 nM fluorogenic probe, and 25 μl of Universal PCR Master Mix (KAPA PROBE Fast qPCR Kit; Kapa Biosystems Inc., Wilmington, MA, USA). The thermal cycling parameters were incubation step at 50 °C for 2 min and incubation step 95 °C for 10 min, followed by 55 cycles of denaturation at 95 °C for 15 s and annealing at 60°C for 1 min. A control containing both no template cDNA mRNA tubes indicate negative amplification. The following probe spans exons assays (all obtained from Applied Biosystems) were amplified: CGRP (*Calca*, Mm00801463_g1), VEGF-A (*Vegfa*, Mm00437304_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (*Gadph*, Mm99999915_g1; TaqMan® Rodent GAPDH Control Reagents with the VIC probe). The threshold cycle (Ct), defined as the cycle at which amplification of the PCR product enters the exponential phase was determined for each gene by plotting the fluorescence level versus the cycle number on a logarithmic scale. The relative expression levels of the genes of interest (*Calca, VEGF-A* were estimated by calculating the DCt value, defined as the difference in the Ct values of the targets and the reference gene (*GAPDH*). The ΔCt value was inversely proportional to the level of each mRNA transcript present in mouse lung samples. A higher Ct value corresponded to a lower mRNA level. The levels of amplified mouse cDNAs were expressed as the quantity divided by the value of the same gene at E12.5 on the Y-axis of each graph.

In situ **hybridization.** DNA fragments of CGRP (GenBank accession number NM_001033954.3) and VEGF-A (GenBank accession number NM_009505.4) were subcloned into the pGEMT-Easy vector (Promega Corporation, Madison, WI, USA) to generate sense or antisense RNA probes. All protocols for *in situ* hybridization was undergo Sawada methods (Sawada *et al*., 2022). Colorimetric reactions were performed overnight with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate solution (Sigma-Aldrich Corporation, St. Louis, MO, USA). Every other serial section was also stained with hematoxylin and eosin. The stained sections were examined under an optical microscope (DM-2500; Leica Microsystems GmbH).

Statistical analyses. Differences in the qRT-PCR data among the groups were assessed using two-way analysis of variance followed by Bonferroni's post-hoc test with one categorical independent variable, which consisted of any number of groups, and one continuous variable. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 23.0. (IBM Corporation,

Armonk, NY, USA). For the rate measurement, a positive reaction area (20 mm per square, n=30) was randomly selected in each stage lung. We analyzed the reactions in each section using image analysis software (WinROOF, Mitani Co., Tokyo, Japan) on a computer based on the measurement methods reported by Sawada methods (Sawada *et al*., 2022) with some modifications. Antisense CGRP mRNA level (%; per 20 mm square) in the mouse lung from E12.5 to P5. The average positive intensity (optical density, OD) was based on the extent of staining (>1999 OD).

RESULTS

mRNA abundance in mouse lungs determined by qRT-PCR. The mRNA abundances of CGRP and VEGF-A were detected in the mouse lung from E12.5 to P5, as shown in Figure 1. CGRP mRNA levels in the lung tissues increased from E12.5 to P5 with the highest level at E17.5 ($P < 0.01$) (Fig. 1a). VEGF-A mRNA levels gradually also increased from E12.5 to E17.5, with the highest level at P5 ($P < 0.01$) (Fig. 1b).

In situ hybridization in the lung. At E12.5, numerous antisense probes for CGRP-positive cells were scattered around the mesenchyme cells, forming numerous amorphous luminal structures (Fig. 2e). The antisense probe for VEGF-A-positive cells was also found in almost the same site (Fig. 2f). At E14.5, the antisense probe for CGRP mRNA was concentrated in the inner side of the tall and columnar epithelium around the pseudoglandular structures (Fig. 2h), whereas that for VEGF-A mRNA was mainly scattered in whole epithelium cells (Fig. 2i). At E17.5, the antisense probe for CGRP mRNA formed a cluster at the inner side of epithelium cells of the canalicular and saccular structures (Fig. 2k), whereas that for VEGF-A mRNA was detected almost entirely throughout at the inner side of the same site and around connective tissues (Fig. 2l). At P0, the antisense probe for CGRP mRNA was found in a few clusters of epithelium cells (Fig. 2n), whereas that for VEGF-A mRNA was found in the saccular epithelium and other connective tissues (Fig. 2o). At P5, the antisense probe for CGRP mRNA was detected in the cluster epithelium cells of the alveoli (Fig. 2q), whereas that for VEGF-A mRNA was detected to be scattered in whole lungs (Fig. 2r). Figures 2a, d, g, j, m and p were shown by the hematoxylin and eosin staining for the counterstain at each stage.

The ratio of CGRP mRNA expression levels of lung. The expression levels of the CGRP mRNA were quickly increased from E17.5 in contrast to that of whole lung was high expression in E12.5 and E14.5 and almost no detected from E17.5 (Fig. 1c).

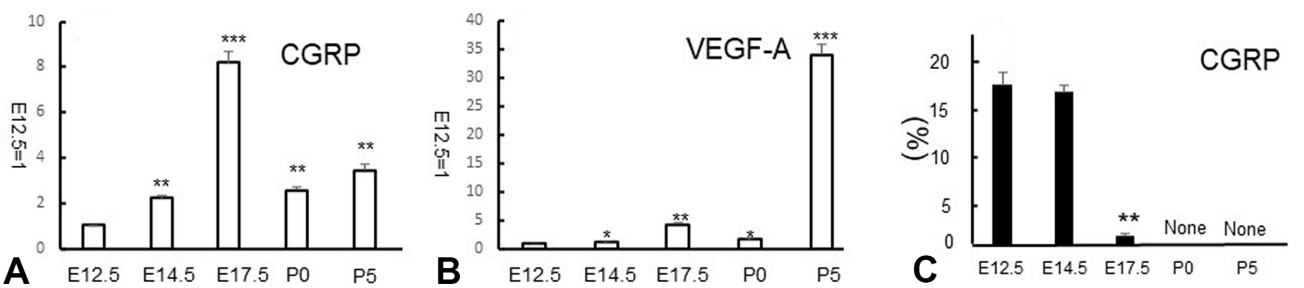

Fig. 1. CGRP and VEGF-A mRNA expression levels at E12.5, E14.5, E17.5, P0, and P5. The abundance of calcitonin gene-related peptide (A, CGRP) and vascular endothelial growth factor (B, VEGF-A) mRNAs were normalized to that of GAPDH at E12.5, E14.5, E17.5, P0, and P5. The ratio of CGRP mRNA expression levels (C, %) of respiratory bronchiole in lung *P < 0.05, **P < 0.01, ***P < 0.001

DISCUSSION

CGRP is reportedly involved in the development of alveoli and bronchioles, the specific roles of which indicate that CGRP participates in the regulation of sensory nerves, blood vessels, and the bronchiole during lung formation (Gontan *et al*., 2008). CGRP is expressed in the trachea and smooth muscle of the lung (Corcoran, 1996). However, it is unclear why its expression increased in the early stage but decreased in the later stages of lung formation in our study. The CGRP and VEGF-A mRNAs expression levels increased at E17.5 during lung formation, particularly in the alveolar progenitor cells and the respiratory bronchioles, similar to VEGF-A mRNA expression identified by *in situ* hybridization in our study. Our results suggest that CGRP is involved in the development of respiratory bronchioles and

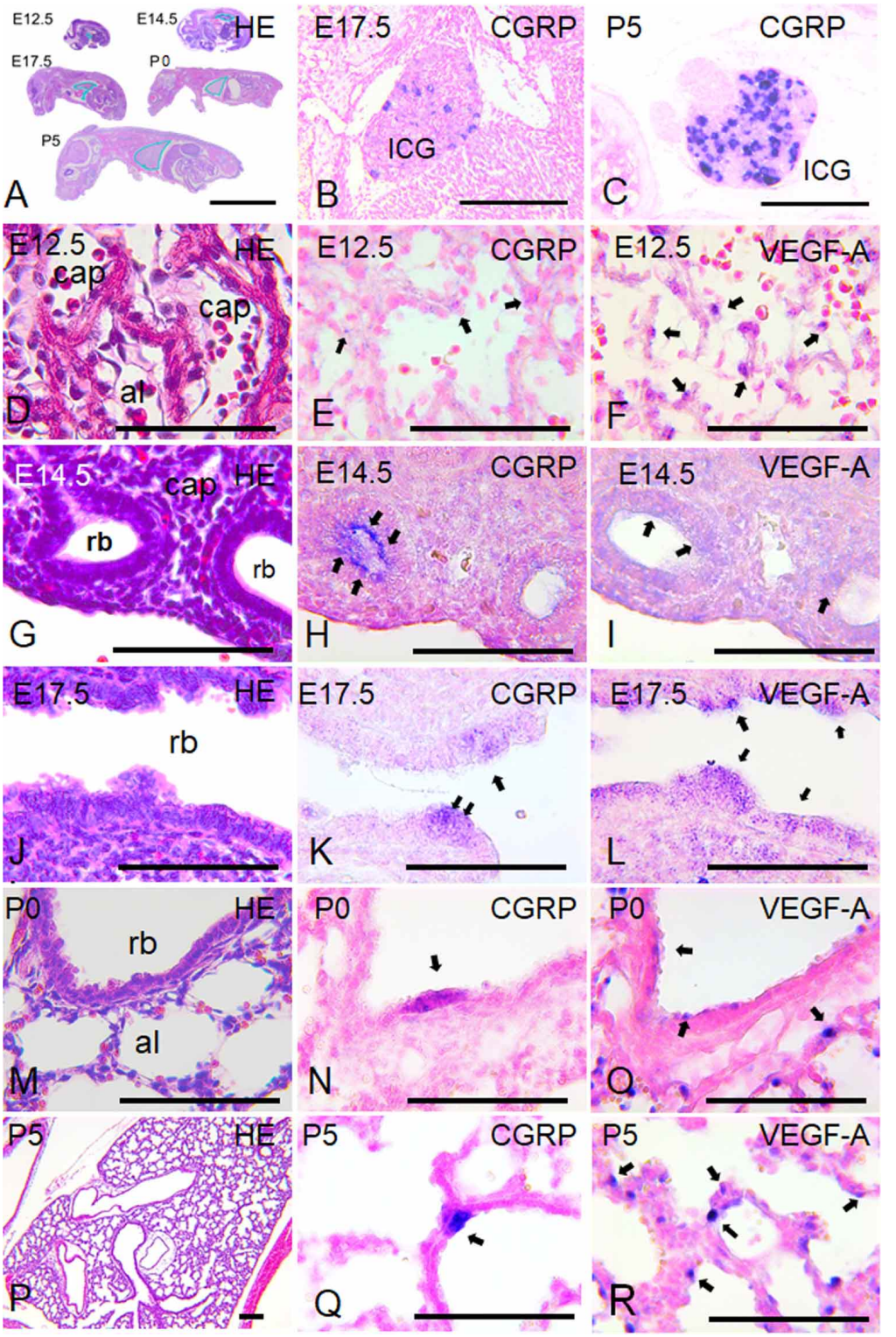

Fig. 2. CGRP (B, C, E. H. K. N. Q) and VEGF-A (F, I, L, O, R) mRNA expression and hematoxylin and eosin staining (A, D, G, J. M, P) of mouse lungs and inferior cervical thoracic ganglia (ICG) (B, C) from E12.5 to E17.5. Sagittal section of mouse by hematoxylin and eosin staining (A). An antisense RNA probe coding for CGRP mRNA was detected in the lung at E12.5 (E), E14.5 (H), E17.5 (K), P0 (N) and P5 (Q) (arrows),An antisense RNA probe coding for VEGF-A mRNA was also detected in the lung at E12.5 (F), E14.5 (I), E17.5 (L), P0 (O) and P5 (R). The CGRP mRNA in ICG was strongly detected at E17.5 (B) and P5 (C) for positive control. cap, capillary vessel; al, alveolus; rb, respiratory bronchiole, A, Bar = 1cm; B-R, Bar = 100 μm;

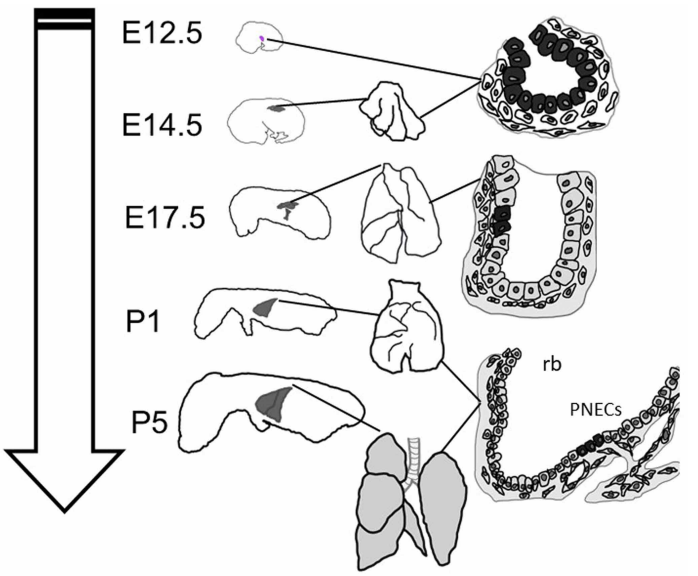

Fig. 3. Schema of lung development. The CGRP was scattered and observed at the inner epithelium of the respiratory bronchiole at from E12.5 to P5. CGRP (gray color) located in the pulmonary neuroendocrine cells from E17.5 were also obtained. rb, respiratory bronchiole

bronchioles. CGRP expression increased at E17.5 during alveolar and bronchial formation, particularly in alveolar progenitor cells and respiratory bronchioles, and that of VEGF-A expression also relatively high at E17.5. However, CGRP expression decreased from the late embryonic stage to the postnatal period, as determined via PCR. The increase in CGRP mRNA from E14.5 suggests that preparations for respiration begin from this period and may promote a substantial increase in blood vessel formation before the postnatal stage. Our *in situ* hybridization analysis revealed a strong positive CGRP expression in the bronchiole epithelial cells at E17.5 and E14.5. The CGRP expression pattern during the embryonic stages were different from those of CGRP and VEGF-A in other organs during development (Sawada *et al*., 2022). Precise temporal and spatial expression of VEGF-A is required for the vascular patterning to develop during lung morphogenesis (Akeson *et al*., 2003). VEGF in the airway epithelial cells is involved in the maturation as well as the proliferation of capillary endothelial cells. CGRP promotes the proliferation and migration of endothelial cells, a function that is associated with VEGF expression during the embryonic stages. Moreover, CGRP is a strong proangiogenic growth factor of lung remodeling, a process mediated by its ability to promote angiogenesis (Tuo *et al*., 2013). CGRP and VEGF-A may co-regulate in a manner similar to that in developing epithelial cells, at least in the embryonic lung from E12.5 to E17.5. Thus, a substantial increase in the CGRP and VEGF-A expression levels during organogenesis is observed during the migration and tube formation of lung epithelial cells during development from E12.5 to E17.5. CGRP produced by ganglion cells may transport to the lung blood vessels, and bronchi via nerve fibers. In addition to ICG, also sensory neurons synthesize CGRP. Many CGRP molecules are transported from the vagal sensory ganglia (jugular nodose complex) in the form of extrinsic CGRP (Springall *et al*., 1987). CGRP promotes the proliferation and migration of endothelial cells along with VEGF expression during the embryonic to postnatal stages, thereby guiding the embryonic, pseudoglandular, canalicular, saccular, and alveolar formation during the development of the respiratory system. Moreover, CGRP and VEGF-A are suggested as important markers that regulate various aspects of the development of the mouse lung formation on E14.5 and E17.5.

CONCLUSION

CGRP promotes the proliferation and migration of endothelial cells with varying VEGF expression during the various stages of development from the embryonic to the fetal stages in the respiratory system. The expression of CGRP and VEGF-A plays a specific role in the morphogenesis of lung development from the embryonic to postnatal stages.

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RESUMEN: El péptido relacionado con el gen de la calcitonina (CGRP) es un neurotransmisor vinculado con la vasculogénesis durante el desarrollo de órganos. El factor de crecimiento endotelial vascular A (VEGF-A) también se requiere para el patrón vascular durante la morfogénesis pulmonar. El CGRP se encuentra principalmente en los órganos y aparece inicialmente en las células neuroendocrinas pulmonares durante la etapa embrionaria temprana del desarrollo pulmonar. Sin embargo, la relación entre CGRP y VEGF-A durante la formación de los pul-

mones sigue sin estar clara. Este estudio investiga las expresiones de ARNm de CGRP y VEGF-A en las etapas embrionaria, pseudoglandular, canalicular, sacular y alveolar del desarrollo pulmonar desde el día embrionario 12,5 (E12,5) hasta el día postnatal 5 (P5) a través de la reacción en cadena de la polimerasa cuantitativa en tiempo real. (qRT-PCR) e hibridación *in situ*. Además, analizamos la expresión de CGRP mediante inmunohistoquímica. El ARNm de VEGF-A se dispersó principalmente por todo parénquima pulmonar desde E12,5. Se encontró que CGRP se expresaba en unas pocas células epiteliales de los bronquiolos canaliculares y respiratorios del pulmón desde E12,5 a P5. Se detectó fuertemente una sonda antisentido para ARNm de CGRP en el pulmón de E14,5 a E17,5. El CGRP endógeno puede regular el desarrollo de los alvéolos embrionarios de E14,5 a E17,5 de manera temporal.

PALABRAS CLAVE: neurotransmisor, péptido relacionado con el gen de la calcitonina, vasculogénesis, embrionario, posnatal, hibridación *in situ*.

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