WIDESPREAD EXPRESSION OF EGFP IN EPITHELIAL TISSUES IN AN E-CADHERIN BAC TRANSGENIC MOUSE LINE

by

ESTEFANIA OLIVAR

(Under the Direction of Brian Condie)

ABSTRACT

E-cadherin, widely expressed in epithelial cell types, plays an important role in cell-cell adhesion in epithelial tissues. Here I characterize a transgenic mouse strain in which EGFP expression is driven from the E-cadherin gene to evaluate its use as a tool. EGFP expression was analyzed in the stromal epithelium of the thymus made up of thymic epithelial cells-TECs. Our results show that high levels of EGFP expression in the thymus is detected exclusively in medullary TECs and correlates with a subset of thymic epithelial cells in the medulla where E-cadherin is expressed highest. We have characterized EGFP expression in other epithelial tissues and have concluded that EGFP expression recapitulates CDH1 expression. We therefore propose that this mouse model can be used as a marker for a subset of medullary TECs allowing them to be distinguished from the rest of the population as well as for epithelial cells in other organs.

INDEX WORDS: E-cadherin, CDH1, Cdh1, EGFP, thymus, medulla, TEC, mTEC, transgenic mouse, GENSAT.

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DEDICATION

I would like to dedicate all my work to my dear family and the love of my life John O'Neil. Everything that has brought me to this point has been made possible by the support and love of my family specially my mother Vivian Carcamo Camargo and my father Eduardo Olivar Pinilla. They have made me the person I am today and I will always be eternally grateful. It was this chapter of my life that make up the contents of this work, which led me to cross paths with John O'Neil for which I am immensely happy and grateful.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

E-cadherin (CDH1): an epithelial cell adhesion protein

The arrangement (and rearrangement) of cells, cell layers, tissues and organs is highly regulated by cell to cell adhesion mediated by the Cadherin family. The cadherins are a super family of receptors that mediate calcium dependent cell to cell adhesion; one of the most common adhesion molecules in this family is E-cadherin (CDH1), named for its prevalence in epithelial tissues [2]. CDH1 is essential in early embryonic stages for pre-implantation of the embryo and in later developmental stages as well as adult stages for maintaining tissue integrity in epithelial tissues. Cell-cell adhesion via CDH1 occurs by homophilic interactions between the extracellular compartments of the receptors in two separate cells that dimerize anchoring those two cells together [Figure 1] [1-3]. CDH1 has also been reported to form heterophilic interactions with other molecules but at lower affinities [3].

Most organs containing epithelial tissues will display the CDH1 marker at the surface of the epithelial cells. CDH1s are also known to interact with β-catenin (among other catenins) on its cytosolic side, linking CDH1 to the actin cytoskeleton and strengthening cell-cell adhesion [4]. The interaction between CDH1 and β-catenin results in sequestering and decreased availability of β-catenin and in this manner indirectly and negatively regulating Wnt signaling in epithelial cells. In Cdh1 deficient mice there is an increase of β-catenin present in the nucleus and an increase of Wnt target genes which is abrogated by the addition of CDH1 [5].

Furthermore studies have hinted to Wnt signaling potentially regulating Cdh1 expression via the Slug gene which acts as a negative regulator of Cdh1 and is a target gene of Wnt signaling.

CDH1 a prominent marker in epithelial tissues including the thymus

The thymus is an epithelial organ that serves as the main site of T-lymphocyte development that home from the bone marrow as immature lymphoid progenitors. Interactions between immature lymphoid progenitors and thymic epithelial cells (among other cell types) results in the differentiation and maturation of progenitors at which point they egress as mature T-cells. The thymus is mainly composed of (thymic) epithelial cells and migrating thymocytes and it is structurally divided into two areas; an outer cortical area composed of cortical TECs and an inner medullary area composed of medullary TECs [6, 7]. The thymic epithelial cells, dendritic cells, vasculature and mesenchymal cells form a complex three dimensional network that facilitates the necessary migration and interactions with thymocytes for their proper maturation [7, 8]. As shown in Figure 2, immature lymphocyte progenitors enter the thymus via vasculature at the CMJ where they migrate through the cortex while they differentiate from DN1 to DN4 to DP, undergo t-cell lineage commitment eventually differentiating into SP in a process called positive selection [9-12]. At this point, SP thymocytes relocate into the medulla where they interact with self-antigens presented by cTECs and DCs; a high affinity or strong interaction between the TCR on the thymocyte and the self-antigens presented by the stromal cells will result in negative selection and apoptosis. Loosely or non-reactive cells survive and differentiate into mature t-cells eventually exiting from the thymus via vasculature [12].

Thymic epithelial cells have been reported to express Cdh1 at particularly high levels in the medullary areas and at very low levels in the cortex [13, 14]. Cdh1 hemophilic interactions play an important role in epithelial organization in the thymus as well as thymocyte

development. Heterophilic interactions between Cdh1 expressed on TECs and the integrin $\alpha_E\beta_7$ expressed on thymocytes are involved in thymocyte development and may lead to thymocyte proliferation [15]. CDH1 is also known to interact with β-catenin and sequestering it in this manner indirectly negatively regulating Wnt signaling in early thymocyte progenitors [16]. As part of the GENSAT project, a reporter BAC transgenic mouse was made that expressed GFP under the control of the Cdh1 promoter. Due to the prominent Cdh1 expression in thymic epithelial cells, we have decided to explore GFP expression in the thymus as means of using it as a marker to identify and isolate TECs.

GENSAT BAC Transgenic Project

The GENSAT project is a large scale project set out to create an atlas of the central nervous system that contains gene expression information using BAC transgenic mice that carry an EGFP reporter gene. A detailed description of the technique and protocol can be found in the paper published by Gong et al in 2003. To summarize the work done in [17] BAC transgenic vectors were modified by introducing an EGFP cassette and a polyA sequence 5'of the native ATG codon [Figure 3]. To ensure EGFP expression reports that of the gene of interest, BACs that covered 50-100 Kb of 5' and 3' flanking intergenic regions were used to ensure appropriate regulatory regions would be included. Several hundreds of genes were chosen to be analyzed including Cdh1 [Table 1 in bold]. Expression of GFP in the transgenic constructs mimics that of the endogenous gene without affecting its expression or function. Circular BAC DNA was introduced into mouse oocytes via pronuclear injections to create founder lines that were screened to choose the optimal line in order to perform a systematic analysis of reporter gene expression in the CNS. Gong et al have demonstrated the functionality of this approach to

reliably analyze gene expression, cell types and migration, and speculate on gene function and roles during development of the CNS [17].

Applications of transgenic GENSAT mice

As part of the large scale analysis of the GENSAT project, the gene expression data was annotated and deposited in a public database (www.gensat.org). The verified library of BAC vectors and transgenic lines created [see table 1 for examples] were made public and accessible so that the scientific community could to use them as a tool in their research and promote advancements in the field of CNS research at a molecular and functional level [17]. Genes analyzed were chosen based on their importance in the CNS and its development. The range of expression for the majority of these genes is not limited to the CNS, and extends to a myriad of cell types, tissues and organs throughout the mouse body. Such expression outside of the CNS was not analyzed in depth by the GENSAT project and has therefore been the subject of research by other labs that use the strain as a tool to analyze their desired gene, cell type, or tissue.

Many of the strains developed by the GENSAT project have been utilized to research gene expression and function at a more detailed level in the CNS and other tissue. Kwon et al use and describe the BAC transgenic strain tg(Eomes-EGFP) created by GENSAT where GFP is expressed under the control of the Eomesodermin (also called Tbr2) promoter. Expression was analyzed in early embryonic stages including at the pre-implantation stage as well as early post natal stages of transgenic mice. EGFP expression was detected in the brain, stomach, pancreas and the placenta. Detailed analysis of BAC transgenic embryos demonstrated that GFP expression closely matches spatiotemporal endogenous eomes expression. Expression of GFP co-localizes with expression of eomes transcript and protein; in addition GFP expression is easily

detected and levels are high. Their analysis suggests that the Tg(Eomes-EGFP) strain is an ideal tool for the study of eomes expressing cells.[18]. This study has demonstrated the usefulness of the GENSAT strains in more than just assaying gene expression patterns. The strains can be used as a tool for identifying and pinpointing specific groups of cells, isolating population of cells and live imaging of cells. Furthermore it demonstrates that EGFP expression is not limited to the central nervous system but rather extends to other tissues where the endogenous Eome gene is expressed.

A similar analysis was performed by Choi et al using the BAC Tg(Prox1-EGFP) mouse where GFP is under the control of the Prox1 promoter. The Prox1 gene is highly expressed on endothelial cells in lymphatic tissues but absent from vascular cells; making the Prox1 driven GFP a fluorescent reporter specific for lymphatic tissues. Detailed analysis of the GFP expression proves that it efficiently recapitulates the expression of the endogenous Prox1. The authors propose the use of this transgenic mouse strain as a tool for in vivo vascular imaging, identification and isolation of lymphatic endothelial cells among other uses based on their GFP expression [19]. This research is an excellent example of the GENSAT strains being utilized completely outside or independently of the CNS field of research providing useful and valuable information.

The Zhang et al group used the transgenic BAC strain Tg(Irx3-EGFP) in combination with other strains in order to better understand the role of Irx3 in the ventricular conduction system or VCS network in the heart. They used GFP expression to identify and isolate Irx3 expressing monocytes from the VCS to characterize expression patterns of other markers in this subset of cells [20]. Similarly, Hall-Glenn et al wanted to understand the role of CCN2 in angiogenesis. Using the transgenic mouse strain Tg (CCN2-EGFP) they were able to determine

the expression pattern of CCN2 in developing embryonic vasculature in order to better understand its role [21].

There are a myriad of strains developed by the GENSAT project that can be used to analyze gene expression and function in different cell types and tissues. One example of the organs that can be analyzed using strains from GENSAT is the thymus. Based on analysis from Griffith et al. [14] the thymus has shared as well as unique gene expression among its different compartments. They have assessed markers that are medulla specific or cortex specific. Table 1 represents genes that have high signal in the medulla (and low signal in the cortex) obtained from Griffith et al. [14] data, for which GENSAT has created a BAC transgenic line. Genes with the highest mean medulla signal would make the best candidate for mTEC specific markers. For example Igfbp4 codes for an insulin growth factor binding protein that inhibits IGF functions. IGFBP is well expressed in the medulla of the thymus and its overexpression leads to disruption of thymic growth [22]. Similar to the Cdh1-EGFP strain, GENSAT has created a strain that carries an EGFP marker under the control of the Igfbp promoter. The gene Fezf2 highly enriched in mTECs according to the data in [14] encodes for a zinc finger transcriptional repressor protein (FEZF2) that is highly expressed and very important during brain development [23]. GENSAT has two different strains that involve Fezf2, one that expresses EGFP and one that expresses Tomato under the Fezf2 promoter. Having two strains to work with expands the possibilities, since they can be used anywhere from understanding expression patterns of FEZF2, to using them for cell isolation and imaging including live imaging. Histidine decarboxylase (HDC) has been shown to be important for hormone balance in immune cells including t-lymphocytes [24]. For HDC, several strains have been created by GENSAT that include EGFP, Tomato under the control of Hdc as well as several cre strains. The glycoprotein Tenascin C

(TNC) has adhesive properties and is part of the extracellular matrix in the thymus and it is highly expressed in the medullary areas [14, 25]. It is involved in anchoring of t-cell progenitors in the thymus, commitment of t-cells and cell migration among other functions not limited to the thymus [25]. GENSAT crated a strain that produces EGFP under the control of the Tnc promoter that can be useful in better understanding not just the function of TNC but the identity of this cell population as well as in imaging them. In addition to the aforementioned strains as well as the tg(CDH1::GFP) strain analyzed here, there are several strains developed by GENSAT that can be used as tools to study and better understand the thymus.

Methods

Table 1 was created by merging microarray data from [14] with the list of strains created by the GENSAT project. The table contains markers that are good medullary marker candidates due to their high medullary signal and low cortical signal. The signal parameters were decided base on the signal obtained for the Cdh1 marker using the central cortical signal for Cdh1 as the cutoff signal so that only markers that had cortical signal equal or lower to 600 would be considered good candidates.

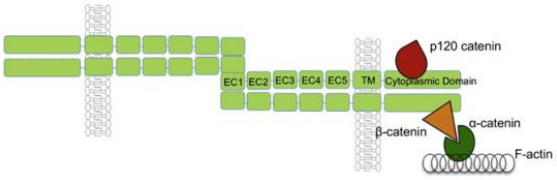

Figure 1. Structure of E-cadherin and illustration of cell-cell adhesion. Adapted from [1]. Extracellular domains made up of cadherin repeats form homophilic bonds. The intracellular domain binds catenins and F-actin cytoskeleton.

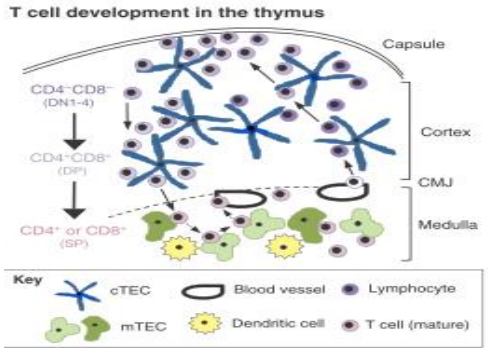

Figure 2. Structure and function of the thymus: T cell development. Adapted from [6]. See text for details. DN1-4, DP and SP, differentiating thymocytes at different development stages within the thymus. See legend for cell types.

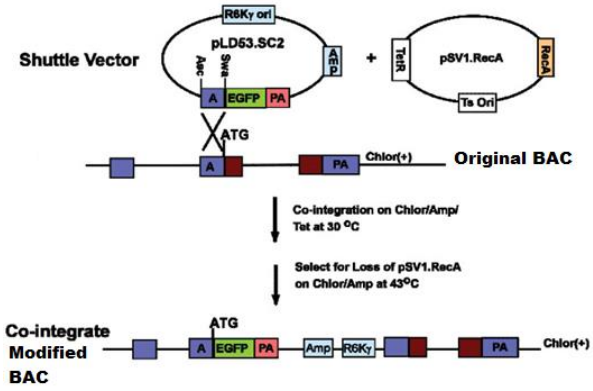

Figure 3. Schematic for BAC modification and general map of modified BACs. Adapted from [14].

Table 1: Modified BACs by GENSAT. Adapted from [6 and 14]. Clusters of genes that are highly expressed in the thymic medulla as found in [14] paired to a corresponding modified transgenic mouse strain candidate made by GENSAT that can be used for analyzing gene expression and function.

Gene Symbol	mean medulla signal	mean perimed cortex signalb	mean central cortex signal	mean subcaps cortex signal	Entrez Gene ID	MMRRC Strain	Marker
Rgs5	11302.5	446.3	341.1	449.9	19737	STOCK Tg(Rgs5-EGFP)JM24Gsat/Mmucd	EGFP
Igfbp4	6825.5	1025.8	220.5	584.8	16010	STOCK Tg(Igfbp4-EGFP)IS47Gsat/Mmucd	EGFP
	3908.0	393.3	264.4	379.2	16010	STOCK Tg(Igfbp4-EGFP)IS47Gsat/Mmucd	EGFP
Igfbp5	6185.1	607.3	273.2	583.1	16011	STOCK Tg(Igfbp5-EGFP)JE168Gsat/Mmucd	EGFP
Cdh1	**5391.7**	**822.8**	**521.3**	**962.7**	**12550**	**STOCK Tg(Cdh1-EGFP)AR201Gsat/Mmucd**	**EGFP**
Calcb	3490.3	80.3	29.2	20.6	116903	STOCK Tg(Calcb-EGFP)PE53Gsat/Mmucd	EGFP
Enpp2	3337.1	1189.1	341.1	218.9	18606	STOCK Tg(Enpp2-EGFP)OR56Gsat/Mmucd	EGFP
Col3a1	2933.1	693.1	426.0	2181.2	12825	STOCK Tg(Col3a1-EGFP)DJ124Gsat/Mmucd	EGFP
Fezf2	2895.1	66.2	17.2	4.4	54713	STOCK Tg(Fezf2-EGFP)CO61Gsat/Mmnc	EGFP
Hdc	2654.9	79.7	17.4	31.4	15186	STOCK Tg(Hdc-cre)IM4Gsat/Mmucd	Cre
Col6a1	2355.8	439.7	214.1	450.8	12833	STOCK Tg(Col6a1-EGFP)JB13Gsat/Mmucd	EGFP
Gas1	1998.1	371.8	315.9	927.2	14451	STOCK Tg(Gas1-EGFP)JT116Gsat/Mmucd	EGFP
Sparcl1	1908.5	342.2	181.9	310.6	13602	STOCK Tg(Sparcl1-EGFP)BE2Gsat/Mmnc	EGFP
Mdk	1844.0	127.2	102.4	190.3	17242	STOCK Tg(Mdk-EGFP)DJ208Gsat/Mmucd	EGFP
Sema6d	1829.4	367.6	311.7	301.6	214968	STOCK Tg(Sema6d-EGFP)EW185Gsat/Mmucd	EGFP
Ccnd1	1740.1	289.7	175.9	160.9	12443	STOCK Tg(Ccnd1-EGFP)FP70Gsat/Mmucd	EGFP
Gja1	1713.5	231.6	176.9	324.3	14609	STOCK Tg(Gja1-EGFP)KB40Gsat/Mmucd	EGFP
Jag1	1633.7	175.7	125.7	140.4	16449	STOCK Tg(Jag1-EGFP)OM21Gsat/Mmucd	EGFP
Ascl1	1629.1	47.2	18.4	17.7	17172	STOCK Tg(Ascl1-cre)ND216Gsat/Mmucd	Cre
						STOCK Tg(Ascl1-cre)ND216Gsat/Mmucd	Cre

						STOCK Tg(Ascl1-EGFP)AU176Gsat/Mmnc	EGFP
Card6	1439.8	1147.4	411.8	252.6	239319	STOCK Tg(Card6-EGFP)HV22Gsat/Mmucd	EGFP
Tbc1d8	1335.7	322.4	267.7	278.9	54610	STOCK Tg(Tbc1d8-EGFP)IB6Gsat/Mmucd	EGFP
Itgb3	1335.4	778.2	139.3	70.0	16416	STOCK Tg(Itgb3-EGFP)IK58Gsat/Mmucd	EGFP
Gda	1276.0	271.3	117.8	223.3	14544	STOCK Tg(Gda-EGFP)LH56Gsat/Mmucd	EGFP
Gpr83	1077.6	348.5	86.4	87.5	14608	STOCK Tg(Gpr83-EGFP)DV46Gsat/Mmucd	EGFP
Igf1	1068.6	586.7	348.7	216.0	16000	STOCK Tg(Igf1-cre)PY2Gsat/Mmucd	Cre
	663.2	479.9	269.3	256.3		STOCK Tg(Igf1-cre)PY2Gsat/Mmucd	Cre
						STOCK Tg(Igf1-EGFP)CO18Gsat/Mmnc	EGFP
	543.8	616.8	348.9	267.1		STOCK Tg(Igf1-cre)PY2Gsat/Mmucd	Cre
						STOCK Tg(Igf1-EGFP)CO18Gsat/Mmnc	EGFP
Fcer1g	1051.2	430.1	327.5	213.5	14127	STOCK Tg(Fcer1g-EGFP)HY299Gsat/Mmucd	EGFP
Rgs4	1043.2	52.1	9.2	23.2	19736	STOCK Tg(Rgs4-EGFP)HS100Gsat/Mmucd	EGFP
Map1lc 3a	1011.2	159.2	170.6	114.0	66734	STOCK Tg(Map1lc3a-EGFP)KX138Gsat/Mmucd	EGFP
Ptn	992.0	183.6	76.4	140.7	19242	STOCK Tg(Ptn-EGFP)HJ32Gsat/Mmucd	EGFP
Csf2rb2	974.2	322.4	209.2	98.2	12984	STOCK Tg(Csf2rb2-EGFP)IF334Gsat/Mmucd	EGFP
Cldn1	967.8	145.4	55.4	53.0	12737	STOCK Tg(Cldn1-EGFP)OR95Gsat/Mmucd	EGFP
Crim1	932.2	152.7	88.5	73.0	50766	STOCK Tg(Crim1-EGFP)LA7Gsat/Mmucd	EGFP
Id1	930.4	90.3	91.8	94.8	15901	STOCK Tg(Id1-EGFP)NJ135Gsat/Mmucd	EGFP
Nts	929.9	35.1	19.4	5.2	67405	STOCK Tg(Nts-cre)RH4Gsat/Mmucd	Cre
Efnb2	898.7	268.3	309.1	246.1	13642	STOCK Tg(Efnb2-EGFP)GY92Gsat/Mmucd	EGFP
Gfra2	897.6	186.2	102.3	54.7	14586	STOCK Tg(Gfra2-EGFP)QX53Gsat/Mmucd	EGFP
Sulf2	871.3	826.9	510.6	540.6	72043	STOCK Tg(Sulf2-EGFP)HQ3Gsat/Mmucd	EGFP
Sfrp1	867.8	186.6	110.4	82.5	20377	STOCK Tg(Sfrp1-EGFP)DH142Gsat/Mmucd	EGFP
Bmpr1a	827.9	570.7	501.6	598.3	12166	STOCK Tg(Bmpr1a-EGFP)ID90Gsat/Mmucd	EGFP
Stx1a	816.4	848.1	502.0	286.3	20907	STOCK Tg(Stx1a-EGFP)FV144Gsat/Mmucd	EGFP
Rhob	775.7	399.5	350.6	405.9	11852	STOCK Tg(Rhob-EGFP)NG172Gsat/Mmucd	EGFP
Sdc3	748.3	463.1	248.8	233.9	20970	STOCK Tg(Sdc3-EGFP)NC124Gsat/Mmucd	EGFP
Plxnb2	735.2	162.6	98.8	57.3	140570	STOCK Tg(Plxnb2-EGFP)JX73Gsat/Mmucd	EGFP

Gpr68	700.1	301.2	173.4	151.4	238377	STOCK Tg(Gpr68-EGFP)IU33Gsat/Mmucd	EGFP
Anxa4	700.1	44.9	15.3	19.6	11746	STOCK Tg(Anxa4-EGFP)ME72Gsat/Mmucd	EGFP
Dtx4	685.8	396.5	341.9	449.5	207521	STOCK Tg(Dtx4-EGFP)HA102Gsat/Mmucd	EGFP
Plekhg1	673.1	298.7	241.4	246.0	213783	STOCK Tg(Plekhg1-EGFP)MS27Gsat/Mmucd	EGFP
Ttr	667.6	36.8	16.3	12.0	22139	STOCK Tg(Ttr-EGFP)NE84Gsat/Mmucd	EGFP
Apod	659.9	262.6	98.4	257.7	11815	STOCK Tg(Apod-EGFP)GN316Gsat/Mmucd	EGFP
Spp1	645.5	57.6	5.7	5.1	20750	STOCK Tg(Spp1-EGFP)PD43Gsat/Mmucd	EGFP
Htr7	641.0	71.4	70.5	50.2	15566	STOCK Tg(Htr7-EGFP)ST29Gsat/Mmucd	EGFP
Trf	636.0	249.9	251.3	296.6	22041	STOCK Tg(Trf-EGFP)IF181Gsat/Mmucd	EGFP
Cryab	586.0	133.1	92.5	141.0	12955	STOCK Tg(Cryab-EGFP)HS240Gsat/Mmucd	EGFP
Ckmt1	583.5	15.3	6.3	16.7	12716	STOCK Tg(Ckmt1-EGFP)JB4Gsat/Mmucd	EGFP
Pcdh17	569.6	69.9	40.1	46.7	219228	STOCK Tg(Pcdh17-EGFP)MR152Gsat/Mmucd	EGFP
Wwc1	564.2	355.9	311.0	350.4	211652	STOCK Tg(Wwc1-EGFP)OJ47Gsat/Mmucd	EGFP
Cd34	547.1	156.9	107.1	168.5	12490	STOCK Tg(Cd34-EGFP)MF6Gsat/Mmucd	EGFP
Pcp4	538.9	32.0	18.9	20.4	18546	STOCK Tg(Pcp4-EGFP)GI332Gsat/Mmucd	EGFP
Pvrl3	537.5	130.0	95.2	112.6	58998	STOCK Tg(Pvrl3-EGFP)HP113Gsat/Mmucd	EGFP
Avil	532.0	16.5	11.2	9.7	11567	STOCK Tg(Avil-EGFP)QD84Gsat/Mmucd	EGFP
Efs	499.2	227.0	221.7	279.1	13644	STOCK Tg(Efs-EGFP)JR5Gsat/Mmucd	EGFP
Grp	497.5	60.9	15.5	30.1	225642	STOCK Tg(Grp-EGFP)DV197Gsat/Mmucd	EGFP
						STOCK Tg(Grp-cre)KH288Gsat/Mmucd	Cre
						STOCK Tg(Grp-cre)KH107Gsat/Mmucd	Cre
Tle1	491.4	324.8	296.6	344.7	21885	STOCK Tg(Tle1-EGFP)IC137Gsat/Mmucd	EGFP
Ptprz1	490.9	283.7	291.1	417.5	19283	STOCK Tg(Ptprz1-EGFP)EN146Gsat/Mmucd	EGFP
Resp18	481.9	2.3	1.9	3.2	19711	STOCK Tg(Resp18-EGFP)FT127Gsat/Mmucd	EGFP
Cxcl11	464.8	120.0	89.8	70.7	56066	STOCK Tg(Cxcl11-cre)KN257Gsat/Mmucd	Cre
						STOCK Tg(Cxcl11-EGFP)HE40Gsat/Mmucd	EGFP
Gng13	454.6	28.7	14.0	30.5	64337	STOCK Tg(Gng13-EGFP)GI206Gsat/Mmucd	EGFP
Anxa4	454.6	30.7	32.9	36.3	11746	STOCK Tg(Anxa4-EGFP)ME72Gsat/Mmucd	EGFP
Chd7	452.8	148.0	42.9	35.7	320790	STOCK Tg(Chd7-EGFP)IE59Gsat/Mmucd	EGFP
Gipc2	440.4	114.6	120.9	101.9	54120	STOCK Tg(Gipc2-EGFP)QS111Gsat/Mmucd	EGFP

Ddr1	437.7	128.9	113.9	161.8	12305	STOCK Tg(Ddr1-EGFP)JD143Gsat/Mmucd	EGFP
Eps8l2	431.7	184.0	185.8	193.3	98845	STOCK Tg(Eps8l2-EGFP)LY20Gsat/Mmucd	EGFP
Cxcl13	429.4	86.7	80.2	102.8	55985	STOCK Tg(Cxcl13-EGFP)SE33Gsat/Mmucd	EGFP
Selm	425.8	18.7	34.2	16.7	114679	STOCK Tg(Selm-EGFP)NE35Gsat/Mmucd	EGFP
Kctd4	419.4	96.3	77.2	78.1	67516	STOCK Tg(Kctd4-EGFP)IB253Gsat/Mmucd	EGFP
Sort1	414.1	296.4	344.3	389.3	20661	STOCK Tg(Sort1-EGFP)EB71Gsat/Mmucd	EGFP
Trim2	404.3	287.9	203.7	180.7	80890	STOCK Tg(Trim2-EGFP)IO71Gsat/Mmucd	EGFP
Sh3bgrl2	403.9	142.2	111.1	116.9	212531	STOCK Tg(Sh3bgrl2-EGFP)QY129Gsat/Mmucd	EGFP
Slc6a4	402.9	28.4	21.1	14.4	15567	STOCK Tg(Slc6a4-EGFP)JP55Gsat/Mmucd	EGFP
						STOCK Tg(Slc6a4-cre/ERT2)EZ8Gsat/Mmucd	Cre-ER
						STOCK Tg(Slc6a4-cre/ERT2)EZ13Gsat/Mmucd	Cre-ER
						B6.FVB(Cg)-Tg(Slc6a4-cre/ERT2)EZ13Gsat/Mmucd: Details,Order	Cre-ER
						STOCK Tg(Slc6a4-cre)ET127Gsat/Mmucd	Cre
						STOCK Tg(Slc6a4-cre)ET33Gsat/Mmucd	Cre
						B6.FVB(Cg)-Tg(Slc6a4-cre)ET33Gsat/Mmucd	Cre
Efnb1	401.4	306.4	326.7	346.8	13641	STOCK Tg(Efnb1-EGFP)GH241Gsat/Mmucd	EGFP
Nr4a2	399.3	140.9	98.6	73.5	18227	STOCK Tg(Nr4a2-EGFP)QU31Gsat/Mmucd	EGFP
Sox9	398.3	118.1	108.2	129.1	20682	STOCK Tg(Sox9-EGFP)EB209Gsat/Mmucd	EGFP
Oxt	398.0	305.8	229.0	195.5	18429	STOCK Tg(Oxt-EGFP)LC317Gsat/Mmucd	EGFP
Thrsp	393.7	20.8	10.3	83.2	21835	STOCK Tg(Thrsp-EGFP)HS276Gsat/Mmucd	EGFP
Adam23	388.2	63.8	43.2	58.5	23792	STOCK Tg(Adam23-EGFP)LR169Gsat/Mmucd	EGFP
Cyb561	387.0	259.1	220.7	359.5	13056	STOCK Tg(Cyb561-EGFP)JI88Gsat/Mmucd	EGFP
Zbtb20	386.6	228.1	191.4	179.1	56490	STOCK Tg(Zbtb20-EGFP)HZ275Gsat/Mmucd	EGFP
Gpc4	382.7	199.3	205.9	311.6	14735	STOCK Tg(Gpc4-EGFP)HZ284Gsat/Mmucd	EGFP
Susd2	372.9	92.6	54.9	39.7	71733	STOCK Tg(Susd2-EGFP)NT201Gsat/Mmucd	EGFP
Nrp2	369.4	170.0	79.8	69.5	18187	STOCK Tg(Nrp2-EGFP)CX13Gsat/Mmmh	EGFP
Nrp2	369.4	170.0	79.8	69.5	18187	STOCK Tg(Nrp2-EGFP)SH25Gsat/Mmucd	EGFP

Igfbp2	368.0	74.5	45.4	52.9	16008	STOCK Tg(Igfbp2-EGFP)JT17Gsat/Mmucd	EGFP
Cdh11	367.6	32.7	25.6	145.3	12552	STOCK Tg(Cdh11-EGFP)DL31Gsat/Mmucd	EGFP
Hes1	363.7	245.0	230.4	450.0	15205	STOCK Tg(Hes1-EGFP)U62Gsat/Mmucd	EGFP
Calca	355.8	29.5	6.4	27.9	12310	STOCK Tg(Calca-EGFP)FG104Gsat/Mmucd	EGFP
Mef2c	347.4	284.7	94.9	137.5	17260	STOCK Tg(Mef2c-EGFP)GJ92Gsat/Mmucd	EGFP

CHAPTER 2

CHARACTERIZATION OF EGFP EXPRESSION IN EPITHELIAL TISSUES IN AN E-CADHERIN BAC TRANSGENIC MOUSE LINE[1]

[1] Olivar, E and B. Condie. To be submitted.

Abstact

The thymus is the main organ of the immune system and it is made up of mostly thymocytes (t-cells) and stromal cells that are mostly epithelial cells, TECs. A well-known marker for epithelial cells is the cell adhesion protein CDH1, also expressed by thymic epithelial cells. We seek to characterze the Cdh1 driven EGFP expression on a transgenic BAC mouse strain in order to develop it as a tool to analyze epithelial cells in the thymus as well as other epithelial tissues. Our analyzsis shows that EGFP expression in the thymus is limited to the medullary compartment and absent from the cortical TECs and t-cells. We also analyzed expression in other predominant epithelial organs including the trachea, esophagus, lungs, kidneny, intestines and skin. EGFP expression recapitulated CDH1 expression in all of these organs allowing the possiblility of using this strain as a tool for analyzing epithelial tissues and cells.

Introduction

CDH1 is a marker characteristic of epithelial cells that aids in the cell to cell adhesion therefore helping to maintain tissue structure. The thymic stroma is mostly composed of thymic epithelial cells organized into different regions; the cortex and the medulla. Thymic epithelial cells in the medulla have been shown to express Cdh1 at very high levels whereas cTECs show very low levels of Cdh1 expression [14]. Such differential expression of Cdh1 allows the use of an CDH1 reporter to identify subsets of different cell types in the thymus. Identification and development of new markers to identify or differentiate populations of cells is still needed in order to better understand the structure and composition of the thymus. The transgenic mouse line TgCdh-EGFP shows EGFP expression wherever CDH1 is expressed in the central nervous system reliably however it remains unknown if the same expression pattern is seen in other epithelial tissues and organs with such reliability. Here we characterze EGFP expression of the

Tg(Cdh::EGFP) mouse in the thymus and other epithilial organs including the trachea, esophagus, lungs, kindney, intestines and skin.

Materials and Methods

Mice

The Cdh1-EGFP transgene was generated from BAC RP23-96N17 by inserting the EGFP coding sequence into the 5' end of the coding sequence. The transgenic mouse stock (Tg(Cdh1-EGFP)AR201Gsat/Mmucd) was obtained from the MMRRC. The mouse stock has been expanded and maintained by crosses to C57Bl/6J mice.

Immunohistochemistry

Postnatal thymus, trachea, esophagus, lung, kidney and intestinal tissues as well as E17.5 embryos were dissected and rinsed in 1X PBS. Tissue samples were fixed in 4% PFA for 1hr at 4°C while E17.5 embryos were fixed in 4% PFA for 10 hours at 4°C. Fixed embryos or postnatal tissues were washed once in 5% sucrose/PBS for 1 hour, once in 15% sucrose/PBS overnight at 4°C and once in 30% sucrose/PBS before embedded and frozen in OCT compound (Sakura Tissue-Tek). Frozen sections of 10um in thickness were cut using a Leica CM3050 S cryostat. Primary antibodies were mixed in 1% donkey serum/PBS and incubated for 3 hrs. at room temperature or overnight at 4°C. After one 10 minute wash with 1X PBS the secondary antibodies diluted in PBS were added to the slides and incubated at room temperature for 30 minutes. Slides were then washed in 1X PBS for 10 minutes three times and mounted in FluoroGel(EMS) and dried overnight at room temperature. For direct fluorescence images of both adult and embryonic stages, sections were washed in 1X PBS for two hours in 15 minute intervals. For direct imaging of post natal thymus sections, slides were washed with 1M

ammonium chloride for 30 minutes followed by washes in 1X PBS for two hours in 15 minute intervals.

The antibodies used in this work include the rabbit anti-GFP polyclonal antibody (Invitrogen, Cat#: A-21311, 1:200), rat anti-CDH1 monoclonal antibody (clone ECCD-2 from Invitrogen, Cat#: 13-1900, 1:400), and biotinylated UEA-1 (Vector Labs, Cat#: B-1065, 1:200). Additional antibodies used were chicken anti-GFP polyclonal antibody (Abcam Cat#: AB13970, 1:800), goat anti-Foxn1 (G-20) polyclonal antibody (Santa Cruz biotechnology Cat#: SC-23566, 1:200) and rabbit polyclonal purified anti-keratin 14 antibody (Covance Cat#: PRB-155P). Secondary antibodies were purchased from Invitrogen.

Results

GFP expression in the thymus detected in medulla

The thymus stroma is mainly composed of thymic epithelial cells that can be subdivided into two compartments; cortex and medulla. Previous studies have reported Cdh1 expression in thymic epithelial cells throughout the thymus with higher concentrations in the medulla compared to the cortex where CDH1 is expressed at very low levels. Immature thymocytes were also reported to express CDH1 during fetal stages and showed reduced expression in postnatal and adult stages [26, 27]. Hence we decided to analyze EGFP expression in the thymus at E17.5 and adult stages. In the thymi of transgene positive mice direct EGFP expression was detected within islets that correspond to the medullary areas identified by the relative cell density visualized by a DAPI stain. [Figure 4]. Because CDH1 is reported in the cortex at low levels, we used an antibody against EGFP to help us detect low EGFP signal and perhaps see expression in the cortex as well as allow us to compare expression against other markers. EGFP was only detected in the medullary islets with no expression at all in the cortex of the thymus.

To better understand the identity and function of the EGFP expressing cells in the medulla we decided to stain thymus sections from transgene positive mice with antibodies specific to thymic epithelial cells (TECs), thymocytes or GFP protein. An antibody against Foxn1, a marker that specifically labels thymic epithelial cells, was used in conjunction with UEA1 and Keratin 14 which both mark subsets of mTECs. Staining with anti-GFP, Foxn1, Keratin 14 and UEA-1 revealed co-localization suggesting the identity of the GFP expressing cells as medullary TECs [Figure 5 and 6]. The population of EGFP+ cells overlapped with UEA-1+ [Figure 5B, C, G] as well as K14+ cells [Figure 5J, K, N] whereas a much smaller subset of EGFP+ cells overlapped with Foxn-1+ cells [Figure 5D, H, L, O and 6J,K]. Overlap among UEA-1+ and EGFP+ regions was significantly greater in the thymus of the E17.5 embryo compared to that of the adult when comparing Figure 5B,C,G to 6F, H or J,L. The majority of the Foxn1+ mTECs were EGFP- (or $EGFP^{low}$) [Figure 5B, D, H, O and Figure 6J, K] whereas the majority of UEA-1+ [Fig 5B, C, G and Fig 6F, H] and K14+ cell sub-populations were EGFP+ [Fig 5F, G].

Co-staining of GFP and Ikaros, a marker for thymocytes, revealed no co-localization of both markers (population is EGFP+Ikaros-) and allowed exclusion of thymocytes from the GFP+ cell population (Fig 6N and 5O). Co-staining of anti-GFP and anti-CDH1 antibodies showed largely overlapping regions of EGFP+CDH1+ cells suggesting that the EGFP expression recapitulates the CDH1 expression in the medulla but not in the cortex [Fig 6F and G]. Staining with the CDH1 antibody revealed differential expression of theCDH1 protein with very low expression levels in the cortex and significantly higher expression seen in medulla. Moreover, EGFP expression in the medulla coincided with very high expression of CDH1 in the same areas in the medulla but absent from the low expressing cortex. Expression of GFP in the medulla is

not equal among all the TECs but rather varies from low to high expression. Age matched transgene negative mice were used as negative controls and no GFP expression was detected throughout the thymus including medullary areas.

GFP expression in the trachea and esophagus

The tracheal lumens is lined with a layer of pseudostratified columnar epithelial tissue that forms a continuous barrier for protection from foreign particles like allergens, pathogens and contaminants [28]. The lumen of the esophagus is lined by stratified squamous epithelial tissue that has a keratinized layer that also serves for protection from external mechanical and chemical damages [29]. Epithelial tissues in both trachea and esophagus express CDH1 at high levels where it plays a very important role in structural and immunological functions [30, 31]. Direct EGFP fluorescence was detected in the tracheal epithelium lining the trachea and the esophageal epithelium lining the esophagus of transgene positive mice [Figure 7D and 8D]. Direct EGFP expression was easily detected and remained homogeneous throughout the entire epithelial area of both the trachea and esophagus unlike the gradient of expression seen on other organs. Co-staining of anti-GFP [Figure 7E and 8I] and CDH1 [Figure 7J and 8J] antibodies showed complete overlap of both antibodies in the epithelial layers of both the trachea and the esophagus showing that EGFP expression recapitulates native CDH1 expression [Figure 7 and 8]. Expression of CDH1 remained homogeneous throughout the entire epithelial layer of both organs without brighter or dimmer sites of expression; this was the same expression pattern for EGFP. Direct EGFP expression in the adult trachea was higher and more defined than that seen in the embryonic E17.5 trachea. Transgene negative mice used as negative controls showed no EGFP expression in either organ all the while antibody detection of CDH1 expression remained normal (data not shown).

EGFP expression in the Kidney.

The kidney tubules and ducts are lined with simple cuboidal epithelial tissue specialized for absorption, secretion and movement of materials [32]. In the developing embryonic kidney, CDH1 is known to be expressed in the ureteric bud epithelium, the renal vesicle epithelium, collecting ducts, the distal tubules and in most tubular epithelium as well as most of the s-shaped bodies [33, 34]. Adult kidney expresses CDH1 the distal tubules, the collecting ducts along with most medullary sections and most of the segments in the nephron at high levels [35]. Due to the differential expression of CDH1 in the kidney of both embryos and adults we wanted to characterize the Cdh1 driven EGFP expression in the kidney. Detection of EGFP using the anti-GFP antibody in adult kidney sections showed high EGFP expression in the collecting ducts around the cortex [Figure 9D]. Co-staining of CDH1 and EGFP antibodies on E17.5 kidney sections showed high and overlapping expression in kidney tubules around the medulla and cortical areas [Fig 9]. EGFP expression can be detected in the collecting ducts (blue arrow) and distal tubules (orange arrow) in the medulla region of the kidney (Fig 9H and L). Expression levels of EGFP also vary among the different structures in the kidney with some areas that express CDH1 and low EGFP but variation is not as drastic as in other organs. EGFP expression appears higher in the structures around the cortex compared to the medulla.

EGFP expression in the Intestines.

Epithelial cells lining the intestines form a single layer of simple columnar epithelial tissue that covers the villi projecting into the lumen as well as the crypt that sit on top of a layer of connective tissue. This epithelium acts as a protective barrier to the harsh environment surrounding the intestines as well as allow absorption and secretion [32]. Epithelial cells in this area express CDH1 at high levels to maintain the tissue structure as well as aid in other processes

like maturation and migration of Paneth and goblet cells [36]. CDH1 is required for the proper development and maintenance of intestinal epithelia in neonates and adult mice [37]. Because CDH1 is so highly expressed in the intestines we decided to analyzed the EGFP expression in the intestines of our transgene positive mice in both embryonic and adult stages. Detection of direct EGPF in the small intestines showed uneven expression of GFP with a small population of cells expressing high levels of GFP in the lining of the intestines of both villi and crypt areas in the duodenum [Figure 10D]. Co-staining of anti-GFP and CDH1 showed overlap of both signals with GFP at base levels as well as a few cells that expressed GFP at very high levels dispersed throughout the organ [Figure 10E and J]. Such high EGFP expressing cells were located in both the tall villi and the crypts of the mucosal layer of the duodenum. The expression pattern was similar in the adult small intestine showing EGFP [Figure 10O] and CDH1 [Figure 10P] expression throughout the villi and crypts with a few brighter EGFP cells throughout the structure.

EGFP expression in the lung.

The air-pockets in the lungs also called alveoli are lined by two types of epithelial cells called alveoli epithelial cells type I and II that express high levels of CDH1. The alveoli are lined by simple squamous epithelial cells that are flat and thin to allow gas exchange [38, 39]. Due to the reported CDH1 expression in the lung we decided to look at EGFP expression in the lungs. Direct EGFP expression was detected in the layer lining the alveoli of the lungs in E17.5 embryos in a tight and homogeneous pattern [Figure 11D]. Co-staining of anti CDH1 [Figure 11E] and EGFP [Figure 11J] antibodies showed complete overlap in the expression of both CDH1 and EGFP. Such complete overlap in the patterns of expression shows that the EGFP expression recapitulates that of the native CDH1.

EGFP expression in the skin.

The skin is made up of an outer layer the epidermis and bottom layer called the dermis made up of connective and adipose tissue. The epidermis is made up of stratified squamous epithelial tissues that contain a wealth of adherence junction formed by CDH1 interactions. CDH1 is expressed in the basal and middle layer of the epidermis but not in the outermost keratinized layer [32, 40, 41]. Expression is also seen in the hair follicles where CDH1 is necessary for the continuous renewal of the hair follicles [42]. CDH1 expression in the skin is important for maintenance of Langerhans cells (LCs) in the epidermis by mediating interaction and attachment of keratinocytes to LCs therefore retaining them in the skin as well as mediating the integrity of the epithelial barrier in the skin [43]. Epithelial cells in the skin show high and homogeneous CDH1 expression [44]. Due to the reported CDH1 expression on the skin we decided to investigate EGFP expression in the skin. Direct EGFP expression was detected in the skin of our transgene positive mice showing high homogenous expression in the epidermis of adult mice [Figure 12D] and in the hair follicles of the transgene positive mice [Figure 12D arrow]. Direct EGFP expression is also detected on whole mount newborn pups allowing for quick genotyping [Figure 12F]. No expression was detected in the epidermis of transgene negative mice [Figure 12B and E] and some autofluoresence from hair is seen in the hair follicles [Figure 12B arrow].

Discussion and Conclusion

The cell adhesion protein CDH1 is expressed by epithelial cells in most major epithelial tissues. We have characterized the EGFP expression of the BAC transgenic Cdh1::EGFP mouse line in order to use it as a tool to analyze epithelial cells and overall tissue. Cdh1 driven EGFP accumulated in the cells allowing easy detection of CDH1 producing epithelial cells. Expression of EGFP did not interfere with native CDH1 expression and development appears normal.

Production of EGFP was detected in several major epithelial tissues including the thymus, trachea, esophagus, lungs, kidneys, intestines and skin.

In the thymus EGPF expression was isolated to medullary epithelial cells while absent from cortical epithelial cells. Staining using an CDH1 antibody showed CDH1 staining both the cortex and the medulla with significantly strong expression in the medulla. Co-staining of CDH1 and EGFP showed that EGFP expression was restricted to areas of very high CDH1 expression located in the medullary areas of the thymus with no EGFP detected in the cortex. This same pattern in observed when viewing GFP expression by direct fluoresence This expression pattern is supported by data from [14] that shows expression of Cdh1 in the cTECs at very low levels but significantly higher in the mTECs explaining why Cdh1 driven EGFP is only detected in the medullary regions. The low amounts of CDH1 expressed in the cortex might not be enough to produce detectable levels of EGFP in the cortex allowing us to only detect EGFP in the medulla where high expression of CDH1 drives high amounts of EGFP that are more than enough for our detection with or without an antibody.

Co-labeling of EGFP and UEA-1 with antibodies showed high overlap between both markers allowign us to identify the population of EGFP expressing cells as UEA-1+ mTECs. In addition, a few cells in the EGFP+ population are negative for UEA-1 as seen in figure 5G white arrow indicating that the EGFP population includes another subset of mTECs that is UEA-1 negative but positive for other markers. We have also detected a few cells that express UEA-1 but do not express EGFP, see figure 5G blue arrow. This absence of EGPF expression could be due to levels of EGFP being too low to detect. This could be atributed to a timing issue, where Cdh1 has been turned on but not enough EGFP has accumulated for us to detect expression, or that Cdh1 expression is low overall and EGFP cannot be detected.

Because not all EGFP+ cells were also UEA-1+ we co-stained with anti-EGFP and anti-K14 to detect a different sub-population of mTECs. Significant overlap was seen between the two markers, identifiying most of the EGFP+ population also positive for K14, see figure 5J, K, N. Similar to the EGFP+ and UEA-1- subpopulation, a small subset of the EGFP+ cells did not express K14, see figure 5N grey arrows. This again indicates that the EGFP population encompases more than one subpopulaiton of mTECs in this case a population that is negative for K14 but positive for other markers like UEA-1. This data indicates that the Cdh1 driven EGFP is a marker for all mTECs including the independent populations of UEA-1+ and K14+ mTECs.

Co-labeling of EGFP and Foxn1 showed that the majority of EGFP+ cells did not seem to express Foxn1. This data seems contradicting to the more accepted knowledge that all TECs (both medullary and cortical) express Foxn1 in embryonic and adult stages [45]. The most likely explanation for our data is that the EGFP+ population does express Foxn1 but that expression is too low for detection via immunohistochemistry apearing as Foxn1- instead of Foxn1^{low}. A better understainding would be gained from performing additional experiments that include cell sorting based on EGFP expression and assaying Foxn1 expression using RT-PCR to detect low Foxn1.

High EGFP expression was seen in the trachea, esophagus, lungs and skin of transgene positive mice in a manner that recapitulates CDH1 expression. Dection of GFP expression in the kidney was only possible using and anti-GFP antibody due to the high fluoresence background of the tissue. Expression was noticeably higher in some structures than others which could be due to a differential expression of CDH1 itself. Nontheless we can conclude that GFP expression recapitulates CDH1 expression in the kideny. Expression of GFP in the intestines was as expexted, lining the outter epithelial layer of the villi. A few very bright “spots” or cells

can be observed where EGFP expression appeared very high. This can be due to higher expression of CDH1 and therefore EGFP or from cells that contain autofluoresent fluids like mucin. The data all together leads us to conclude that EGFP expression recapitualtes CDH1 expression and can be used as a reporter for the identification and isolation of epithelial cells.

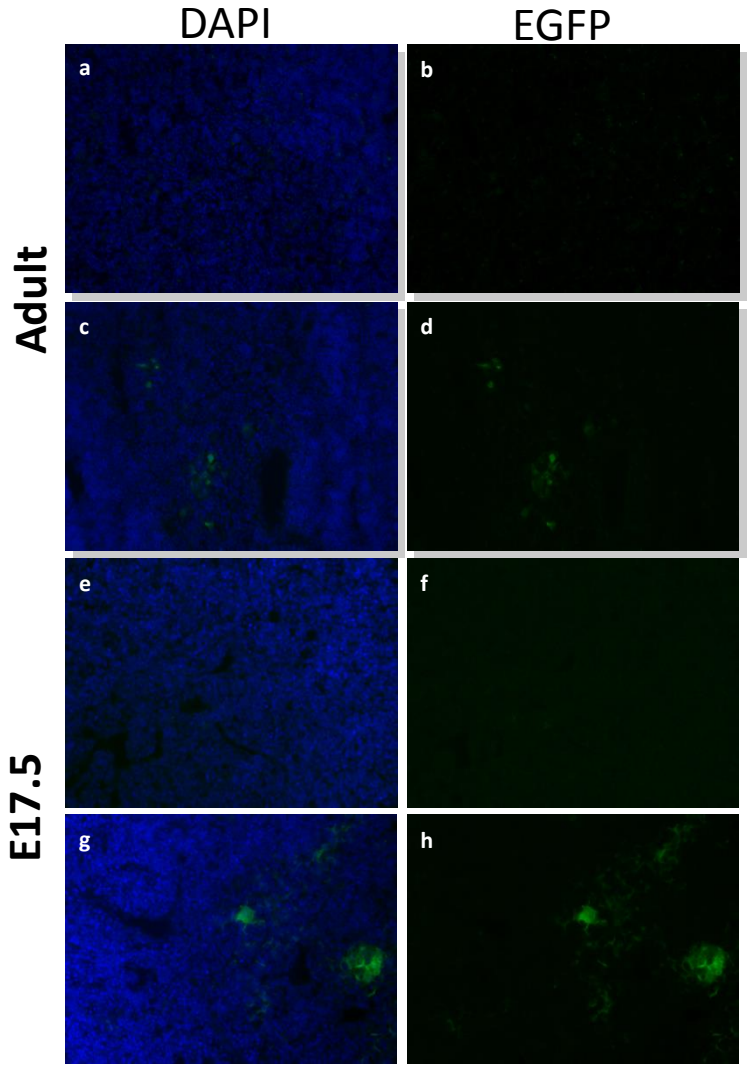

Figure 4. Direct EGFP fluorescence obtained from adult and E17.5 thymus sections. Thymic tissue sections from an adult transgene negative mouse expressing no EGFP (A) Merge including DAPI and (B) green channel showing no EGFP. Thymic sections from adult transgene positive mouse expressing EGFP (C) Merge image showing DAPI and (D) green channel showing EGFP expression in the medulla. Thymus sections from E17.5 transgene negative embryos (E) merge image shwing DAPI and (F) green channel only showing no EGFP expression. Sections of transgene positive embryos (G) Merge image showing DAPI and (H) green channel only showing EGFP expression in the medulla. All images are 20X.

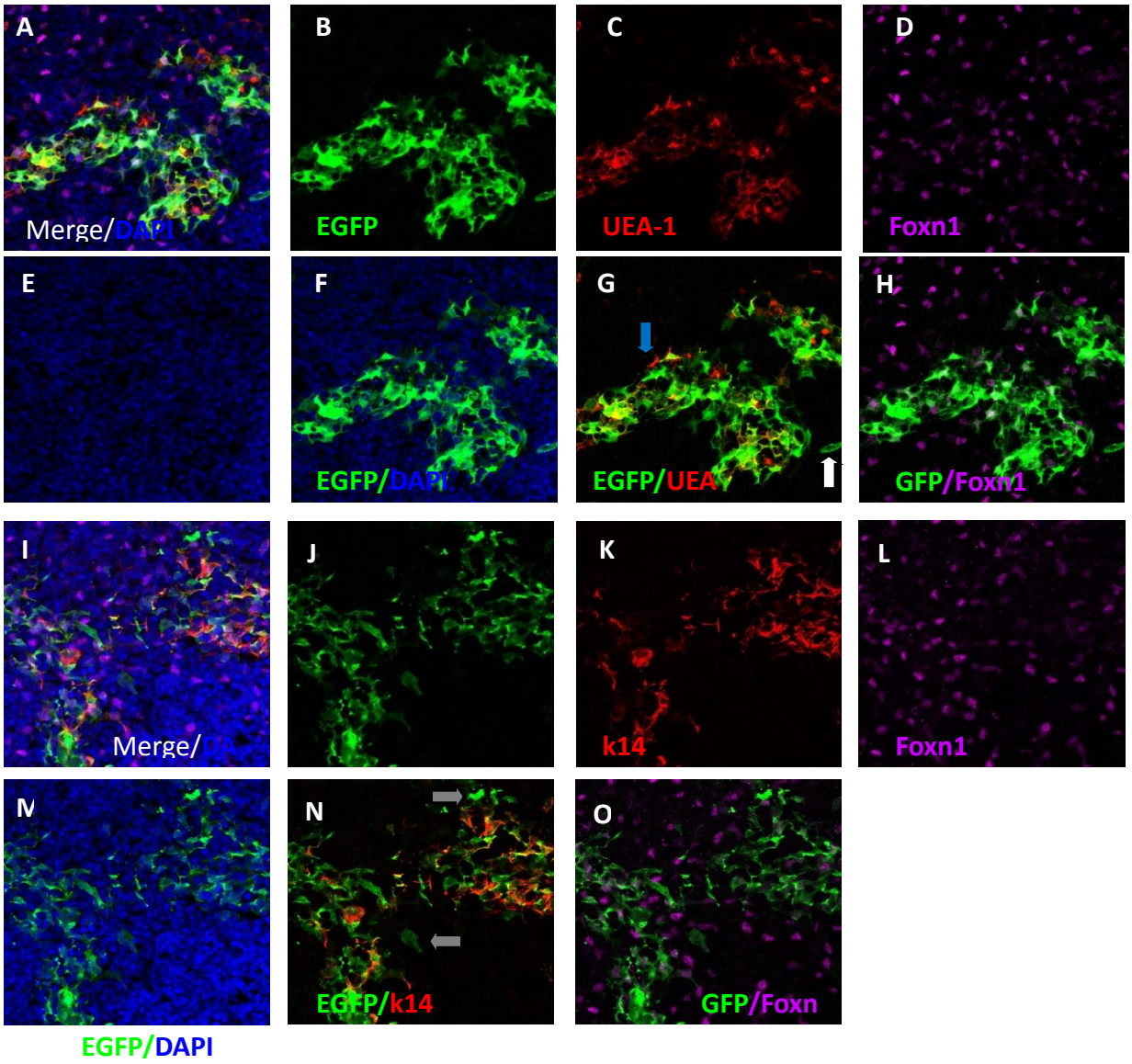

Figure 5: Thymus frozen sections from an E17.5 embryo. A) Merged image showing DAPI in blue. B) anti-EGFP antibody showing high expression in the medulla and non in the cortex. C) anti-UEA-1 antibody showing hign expression in the medulla in the same region as EGFP expression. D) anti-Foxn1 marking TECs in both cortex and medulla. E) Image showing DAPI channel only. F) Merged image showing DAPI in and EGFP in green. G) Merged image showing colocalization of UEA-1 and EGFP. H) Merged image showing colocalization of Foxn1 and EGFP. I) Merged image showing DAPI in blue. J) anti-EGFP. K) anti-Keratin 14 showing expression in TECs in the inner medulla covering the same region as EGFP. L) anti-Foxn1. M) Merged image showing DAPI and EGFP. N) Merged image showing colocalization of K14 and EGFP. O) Merged image showing colocalization of EGFP and Foxn1. All imgaes are high maginification; objective is 40X.

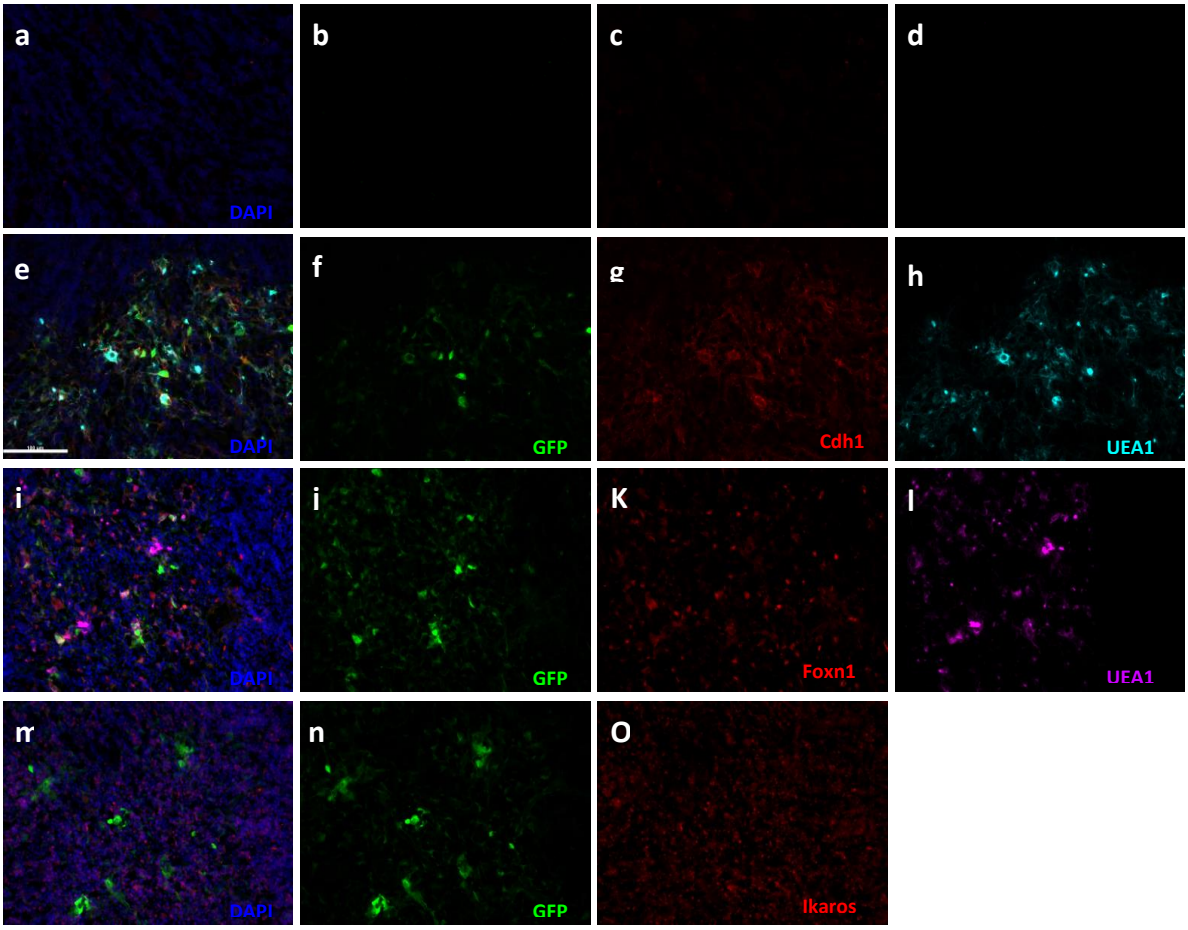

Figure 6: EGFP expression in adult thymus. (A) DAPI stain on a transgene negative control. (B) Green channel showing no EGFP expression on the negative transgene and no antibody control. (C) red channel showing no expression on the negative transgene and no antibody control. (D) Far red channel showing no expression on the negative transgene and no antibody control. Frozen coronal sections from an adult thymus stained with (E) DAPI in blue and merge, (F) anti EGFP (G) CDH1, (H) UEA1, (I) DAPI in blue and merged, (J)anti-GFP, (K)Foxn1, (L) UEA1, (M)DAPI in blue, (N) anti-GFP and (O) Ikaros . Low or no signal was detected in the no-primary controls for all antibodies (A-D). All images are 20X maginification.

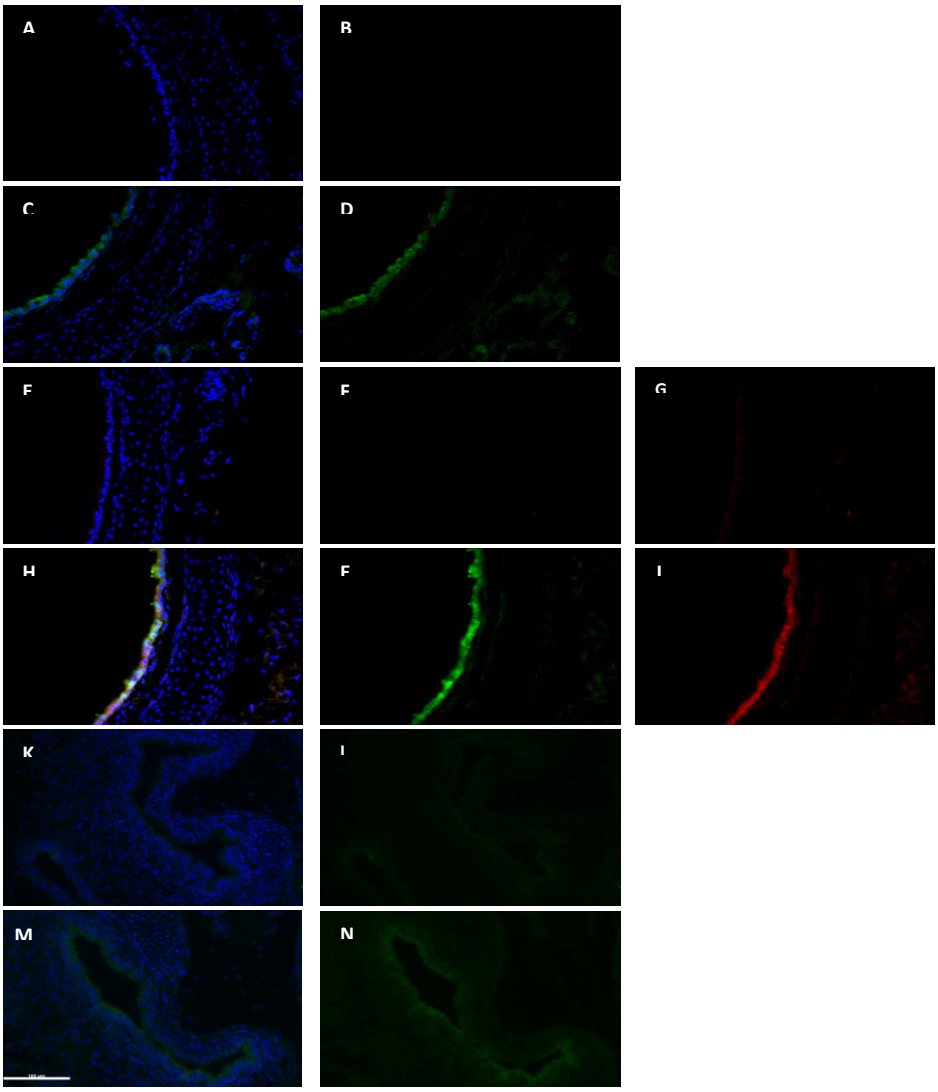

Figure 7. Expression of EGFP in the trachea. Transgene negative control (A) DAPI in blue (B) green channel showing no EGFP expression. Direct EGFP fluorescence in frozen sections of adult trachea (C) DAPI in blue, (D) green channel showing direct EGFP fluorescence. Frozen sections from adult trachea stained with (H) DAPI in blue, (F) no antibody control showing no EGFP expression, (G) no antibody control showing no cdh expression, (H) DAPI in blue (E) anti-GFP, (J) Cdh1 antibodies. (M-N) Direct EGFP fluoresence in frozen section of fetal trachea at E17.5. Low or no signal was detected in the no antibody and negative controls controls (A-B), (E-G) and (K-L). All images are 20X maginification.

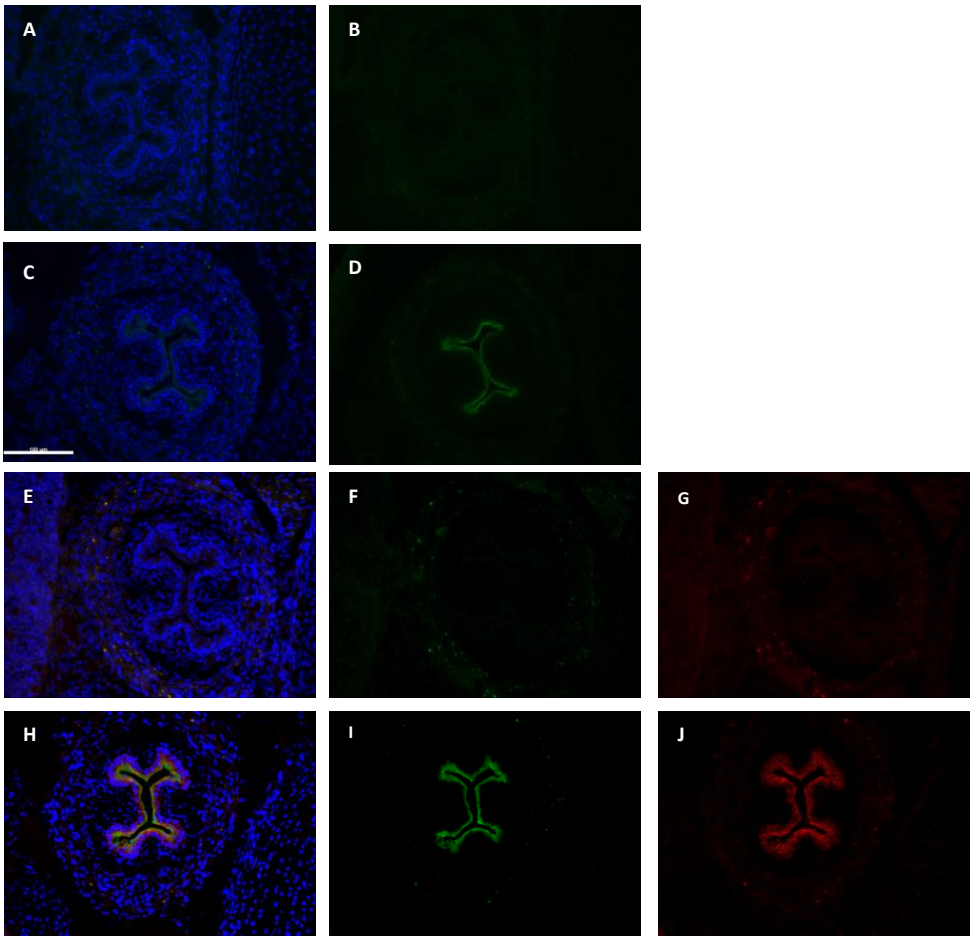

Figure 8. Expression of EGFP in the fetal esophagus.(A) transgene negative showing DAPI. (B) Transgene negative control showing no GFP. (C) Transgene positive showing DAPI (D) Transgene positive showing direct EGFP fluorescence in E17.5 embryos (E) Transgene negative no antibody control showing (E) DAPI, (F) no EGFP expression (G) no CDH1 expression. Frozen transverse sections from E17.5 embryos (H) merge image showing DAPI in blue, (I) anti-EGFP (J) CDH1 expression. Negative and no primary controls showed low or no expression (A-B) and (I-G).

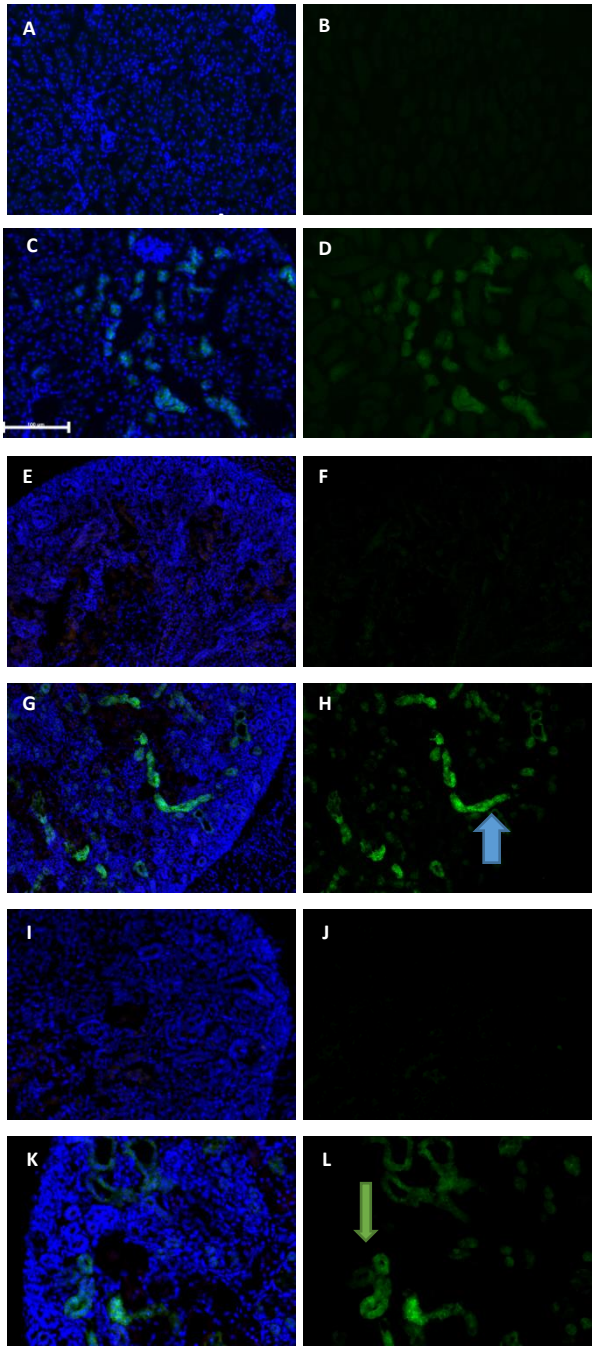

Figure 9. Expression of EGFP in the kidney. Frozen sections of adult kidney.(A-B) negative no primary controls of adult kidney showing no EGFP expression. (C-D) frozen adult kidney sections stained with anti-EGFP antibody. (E-F)No primary control showeing no EGFP expression at 10x of frozen transverse sections from E17.5 embryos. (G-H) Frozen transverse sections from E17.5 embryos stained with anti-GFP antibody at 10x. Arrow pointing to collecting ducts (I-J) No primary control showing no EGFP expression at 20x of frozen transverse sections from E17.5 embryos. (K-L) Frozen transverse sections from E17.5 embryos stained with anti-GFP antibody at 20x. Orange arrow pointing to distal (renal) tubules.

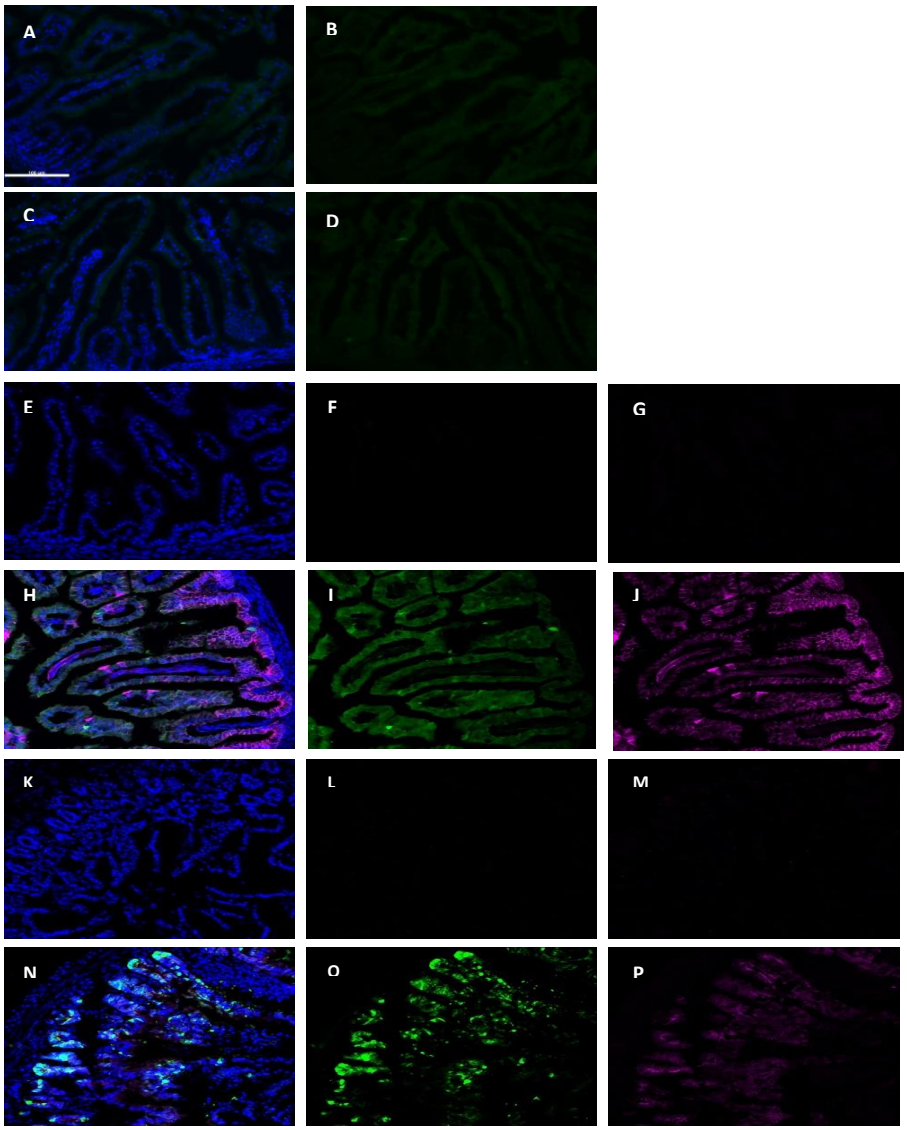

Figure 10. Expression of EGFP in the intestines. Direct fluorescence obtained from frozen transverse sections of E17.5 embryos. (A) Transgene negative embryo showing DAPI in blue, (B) green channel showing no EGFP expression. (C) Transgene positive embryo showing DAPI in blue, (D) green channel showing low EGPF expression in a few cells. Frozen sections from E17.5 embryos stained anti-EGFP and CDH1 (E) DAPI and no primary antibody controls (F) green channel showing no EGFP expression (G) Far red channel showing no CDH1 expression (H) merged image showing DAPI in blue, EGFP in green and CDH1 in red (I) green channel showing EGFP expression using anti-EGFP and (J) Far red channel showing CDH1 expression in purple using anti-CDH1 antibody. Frozen transverse sections of adult small intestine (duodenum). Transgene negative mice showing (K) DAPI in blue, (L) green channel showing no EGFP expression (M) far red channel showing no CDH1 expression. Transgene positive mice showing (N) merged image showing DAPI in blue, (O) green channel showing EGFP expression using anti-EGFP antibody and (P) far red channel showing CDH1 expression in purple using anti-CDH1 antibody.

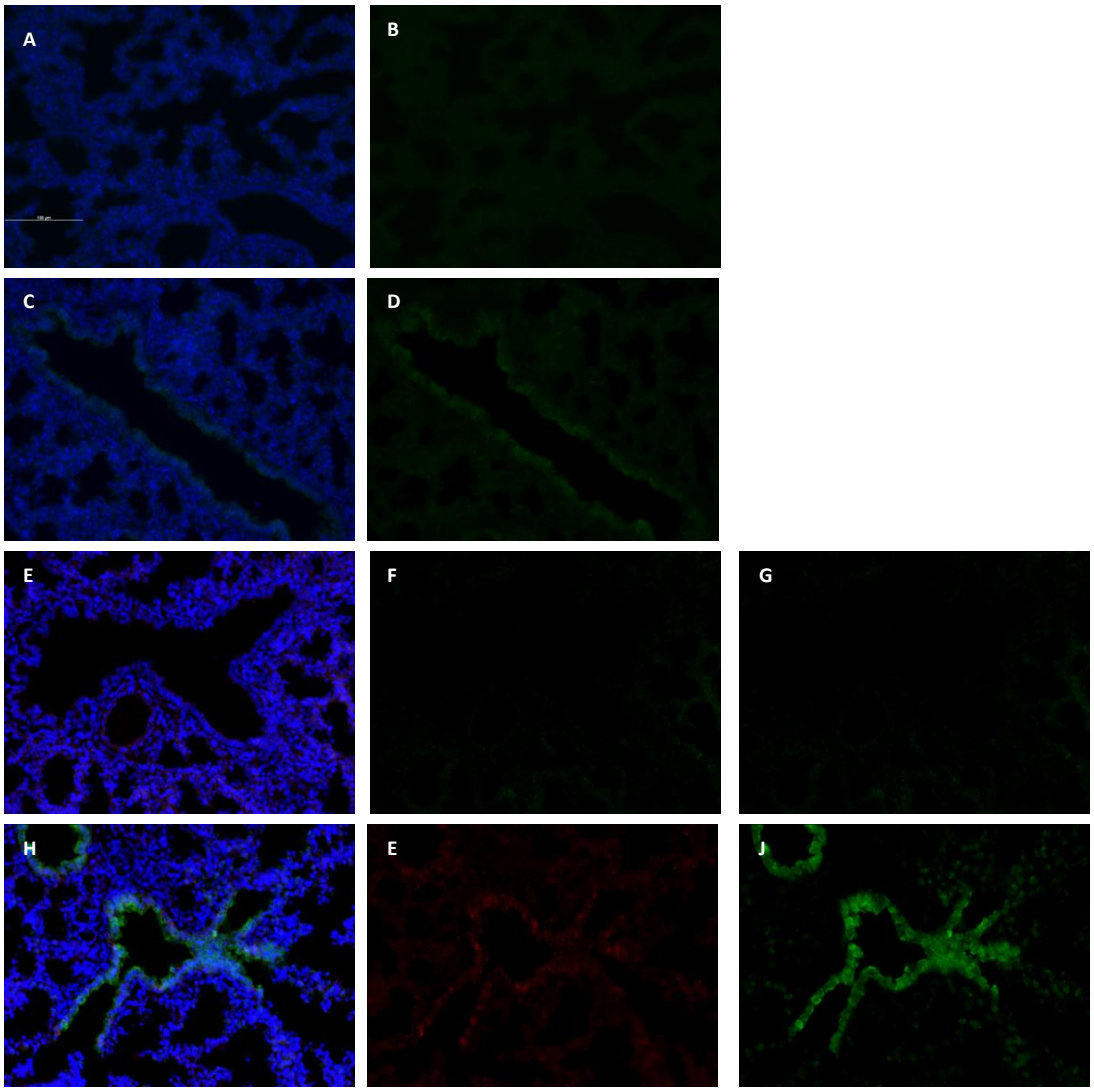

Figure 11. Expression of EGFP in the lung. Lung tissue transverse sections from an E17.5 embryo. (A-B) transgene negative control showing no EGFP expression. (C-D) transgene positive embryo showing direct EGFP fluorescence. (E-G) no primary controls showing no EGFP expression. (H-J) sections stained with anti-GFP and anti-CDH1 antibodies.

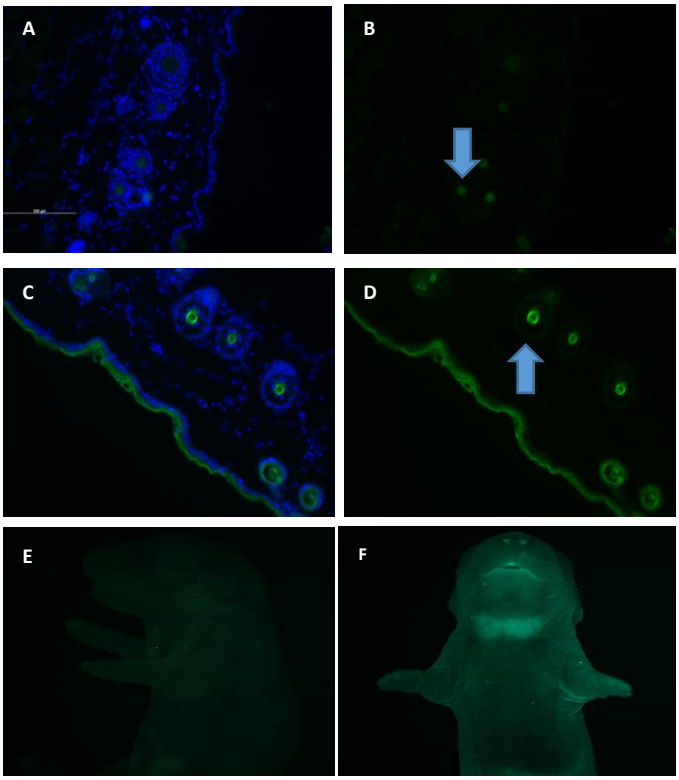

Figure 12. Expression of EGFP in the Skin. Direct EGFP fluorescence obtained from adult skin sections. Adult transgene negative mouse showing (A) merged image with DAPI in blues and (B) green channel showing no EGFP expression. Adult transgene positive mouse skin section showing (C) Merged image showing DAPI in blue and EGFP in green and (D) green channel showing direct EGFP fluorescence. All images are 20X magnification

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