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Tmem121 A Novel Gene Associated With The Proliferation Activity Of Adrenocortical Stem / Progenitor Cells.

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ABSTRACT

Several studies identified the proliferation activity of capsule and subcapsular areas of the adrenal cortex. The centripetal migration of the adrenocortical cells also support the highly proliferative cells which migrate from the region of the adrenal sub-capsular area toward the cortex-medulla boundary were cells undergo apoptosis. These activities associated with the existence of a proliferative stem/progenitor cells niche. The mRNA expression of a novel transmembrane gene (Tmem121) was detected in the adrenal using RT-PCR. The 500bp cDNA amplicon of the Tmem121was cloned into pGEM®-T Easy vector followed by extended PCR reaction which included M13 primers to amplify the target sequence of Tmem121 up-streamed with T7 or SP6 promoter sequence. These amplicons were used as templets for in vitro transcription reaction in the presence of digoxigenin-UTP in order to produce Tmem121 RNA probes which were used for *in situ* hybridization (ISH). This assay was followed by immunostaining assays to investigate the spatial expression of this protein. The results of ISH assays showed the spatial expression of this gene in the capsule and sub-capsular areas of the adrenal cortex which proposed a role of this gene in the proliferation activity of stem/progenitor cells. These findings were confirmed using immunohistofluorescent tests against Tmem121 protein which revealed similar distribution in the capsule and sub-capsular areas of the adrenal gland. Dual immunohistofluorescent experiments showed 72% co-expression of Tmem121 with the proliferation marker Ki67 on the adrenal sections. These findings suggested a propagating role of Tmem121 in the adrenal cortex. However, the suggested propagating effect of Tmem121 required further investigations to specify the association level of the Tmem121expression with the proliferation activity of adrenocortical cells.

Keywords: Tmem121, Ki67, adrenocortical, stem/progenitor cells.

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INTRODUCTION

The adrenal cortex, which is the subject of this study is described as a small endocrine gland, which is composed of three different zones surrounded by a capsule. The first zone underneath the capsule is the zona Glomerulosa (zG), this is responsible for producing and releasing mineralocorticoids (Norris and Carr, 2013). The next zone is the zona Fasciculata (zF), which produces glucocorticoids (Lager, 1991). The final, inner, zone is called the zona Reticularis (zR) which is responsible for releasing dehydroepiandrosterone (DHEA) and the sulphated dehydroepiandrosterone (DHEAS) in humans and other primates, which is also known as adrenal androgen (van Weerden et al., 1992).

Although these zones have distinct occupations, they have renewal capacity in response to physiological influences. The continuously replenished cells suggesting the existence of stem cells that is required for cortical homeostasis (Pihlajoki et al., 2015). Several studies on adrenal cortical stem cells have investigated the capsule, not only as a simple layer that supports the cortex to integrate the gland structure as most histological studies described but also as a niche for adrenocortical stem cells that serve as originator cells for other cells in the cortex (Walczak and Hammer, 2015). The proposed existence of stem and progenitor cells within the adrenal cortex date back to early studies of the 1930s. These studies revealed the capacity of the capsule and sub-capsular layers to restore the adrenal cortex (Ingle and Higgins, 1938). The adrenal cortex of several mammalian species have been suggested to have a mix of clusters of undifferentiated cells in the sub-capsular layer along with cells expressing Cyp11B2 within the zona glomerulosa which are known to have limited steroidogenic capacity in mice (Heikkila et al., 2002) and humans (Nishimoto et al., 2010). These findings suggest the presence of a stem/progenitors cells population in the adrenal cortex. A recent study showed Un-differentiated population of adrenal cortex were isolated and characterized as a stem cell population which were genetically forced to differentiate (Al-Bedhawi 2018). Different studies have shown that proliferating activity were relatively high in the outermost zone of the cortex close to the sub-capsular region (Chang et al., 2013). Lineage tracing study revealed the proliferation and migration of sub-capsular cells toward the differentiated zones of the adrenal cortex starting from zG to the zF (Freedman et al., 2013).

Tmem121 is a poorly studied protein. However, Tmem family members are highly conserved between species and are highly expressed in most embryonic tissues (Zhou et al., 2005), which might involve in the characteristic of stem cells. A human wound healing study showed that Tmem121 is one of the genes that contribute to cell proliferation and migration in human endothelial cells and its expression was co-expressed with the endothelial marker, CD31. Therefore, these results suggested that it might have a potential role in skin wound healing (Bronneke et al., 2015).

MATERIALS AND METHOD

All experiments were conducted in agreement with the guidelines and regulations of the Committee on Experiments of the School of biomedical sciences, University of Reading.

Animals

All the adrenal tissues used in this study were freshly isolated from adult Wistar-rats (weight 250-350g) from Harlan Envigo UK were housed in rooms where the ambient temperature and light were under automatic control. The rats were treated under the United Kingdom Home Office Animals Act 1986. Rats were euthanised by CO_2 asphyxiation.

RNA isolation from rat adrenal tissue

TRIzol reagent (Ambion) was used for the isolation of RNA from rat adrenals. One ml of TRIzol reagent was used for each adrenal gland and homogenised. Samples were centrifuged at 12,000 g for 10 min at 4°C before the addition of 0.1 ml of bromochloropropane (BCP). The supernatant was then transferred to a clean tube. Each sample was then mixed by inversion for 15 seconds and incubated at room temperature for 15 min. Samples were then centrifuged for 15 min at 12,000 g at 4°C and placed directly into a cooling rack. The colourless top layer that contained the RNA was carefully transferred into a clean 1.5ml microcentrifuge tubes before the addition of 0.5 ml of isopropanol. Tubes were mixed by inversion and left to stand at room temperature for 15 min before centrifugation at 12,000g for 25 min at 4°C (the precipitated RNAwas washed

with 0.5 ml of 75% (v/v) ethanol. Then samples were centrifuged at 7,500g for 5 min at 4°C. The ethanol was discarded and the pellet was suspended in 50µl of dionised distilled water (DDW). The genomic DNA contamination was treated with DNase following the provider procedure. The RNA was then re-purified with the above procedure using proportionally less of each reagent.

RNA electrophoresis

The isolated RNA was analysed by agarose gel electrophoresis to test its integrity. The gel was visualised by a transilluminator to identify the ribosomal RNA 28 subunit (28s) and 18 subunits (18s) RNA bands, to confirm RNA integrity.

cDNA synthesis and RT-PCR

For synthesis of total cDNA, a RevertAid First Strand cDNA synthesis kit was used. An aliquot of 1-5µg of isolated RNA was mixed with 1µl (100µM) oligo (dT)18 primer. The volume was made up to 12 µl by the addition of nuclease-free water. Subsequently, the reaction volume was made up to 20µl by adding 4µl of 5X reaction buffer, 1µl of (20u/µl) of RiboLock RNase Inhibitor, 2µl (10 mM) deoxy-dNTP Mix and finally 1µl of (200u/µl) RevertAid reverse transcriptase. The reaction was incubated at 42°C for 1 hour, before termination at 70°C for 5 minutes.

PCR was used to detect the expression of the gene of interest, as well as amplifying the desired amplicons that can be used later for generating RNA probes. Pre-aliquoted Extensor Hi-Fidelity PCR master mix(12.5µl), 1µl of each primer (10µM) Forward primer 5`-3 TTGGTAGGCGATGTGTGCTTCCTG which positioned in the sequence (393-416) and Reverse primer5`-3` ATCATCTTCTGCGGTGCGATGTGC which positioned in the sequence (901-878) were mixed with cDNA (20ng/µl) and 9.5µl of nuclease-free water to make the total volume 25µl. The PCR conditions used were as follows: 94°C for 1 min for initial denaturation and activation of Taq polymerase, followed by 29 cycles of 94°C for 20 sec for denaturation, 58°C for 20 sec for primer annealing, finally the extension was performed at 72°C for 1 min. the same protocol was used with the control target NR5a1 using Forward primer 5`-3 AATTCTCCTTCCGTTCAGCGGACG which positioned in the sequence (113-136) and Reverse primer5`-3` GTTCTCCTGCTTCAAGGCCCG which positioned in the sequence (471-451).

Preparation of RNA-labelled probes for *in situ* hybridisation

Purification of target DNA and cloning into pGEM-T Easy vector

The QIAquick Gel Extraction Kit (Qiagen) was used to purify DNA from agarose gel slices.

The cloning of inserts into the pGEM-T Easy vector (Promega) was conducted by T-A ligation. Ligation reactions were carried out using T4 ligase (Promega) to ligate the genes of interest into the pGEM-T Easy plasmid vector. The reaction mixture was 10µl in total and contained 1µl of 10x ligation buffer. The addition of the DNA insert and the vector was conducted using a 3:1 (insert: vector DNA) molar ratio. Lastly, T4 ligase (1-3 Weiss units) was added. The reactions were mixed by pipetting and incubated overnight at 4°C.

Competent *E. coli* preparation and transformation

The Inoue method for "Ultra Competent" cell preparation and transformation (Inoue *et al*., 1990) was used to prepare Mach1™ *Escherichia coli (E. coli)*.

Aliquots of 50µl of competent cells were thawed on ice before the addition of the ligation reaction mix (10µl). positive and negative controls were prepared in equal volumes for the screening of the targeted colonies.

Purification of complex vector

The blue-white screening was conducted for identifying recombinant bacteria that contained the plasmid of interest. Plasmid DNA was isolated using either the Qiagen Miniprep or the Midiprep purification kit according to the provider's recommendations.

Plasmid sequencing

The plasmids in this project were sequenced in order to verify the targeted nucleotide sequences and to determine the orientation of the inserts in the p-GEM®-T easy vector (Promega) (Appendix B.1). Ten microliters of the purified plasmid (100ng/µl) were submitted to Source BioScience for sequencing in both directions using the appropriate primer sites located in each vector.

Digestion of plasmids with restriction enzymes

Digestion of the plasmid DNA was carried out using EcoRI and their specific buffers from Promega in a total volume of 20µl, which incubated for 2-4 hours at 37ºC in a water bath. The digested DNA was mixed with 6x the loading buffer then profiled by agarose gel electrophoresis.

In vitro transcription to generate the RNA-probes

The orientation of the clone in the pGEM®-T Easy vector was detected following the method previously described by Al-Bedhawi, (2018) knowing the orientation of the insert in the vector is important to generate the right probe. Consequently, PCR experiments were set using M13 primers (forward: CGCCAGGGTTTTCCCAGTCAGC, reverse: TCACACAGGAAACAGCTATGAC) together with primers specific for the inserts for two purposes: firstly, to detect in which direction each insert was ligated to the vector. Secondly, to add the transcription sequence for T7 or SP6 promoters to the inserts, in order to use them later for *in vitro* transcription reaction.

The PCR products were analysed by agarose gel electrophoresis and the resulted bands were extracted and used for *in vitro* transcription.

The +1G of the start codon (ATG) of the RNA polymerase promoter sequence in the DNA template is the first base incorporated into the transcription product (figure 2.2). To make sense RNA, the 5' end of the coding strand (the target strand) must be +1 G of the promoter. For antisense RNA to be transcribed the 5' end of the noncoding strand must be downstream of the +1 G.

Labelled-RNA probes were synthesised to produce two types of Digoxigenin labelled-RNA. The first synthesis, of coding RNA strands (sense probes) were used as a control. The second, of non-coding RNA strands (antisense probe), were used to detect their mRNAs targets in the tissues. These RNA strands were generated using Digoxigenin RNA labelling NTPs mix, which made them detectable probes through an *in vitro* transcription reaction.

In order to prepare the antisense probe for the inserts, an *in vitro* transcription reaction was setup in a 1.5ml microcentrifuge tube with a total reaction volume of 20µl. The mix contained DNA template (200ng), 2µl of 10x Digoxigenin RNA labelling NTPs mix (Roche) and 4µl of the 5x Transcription buffer (Appendix A.18). Finally, 1µl of (20u/µl) RNase inhibitor and 2µl of (20u/µl T7 or SP6) RNA polymerase was added according to the insert orientation in the vector. The total volume of the reaction was made up to 20µl with RNase-free water.

Each reaction was mixed and briefly spun before incubating at 37°C for 2 hours. Finally, 2µl of DNase I RNase-free (1unit/µl) was added and the reaction was incubated for a further 15 minutes. Following transcription, 2µl of the reaction was run on an agarose gel to detect the formation of RNA. The probe concentration was measured by spectrophotometry. Three positive control probes of Sf1 were also prepared to be used for the *in situ* hybridisation experiments.

Ribo-probe cleaning

The Qiagen's RNeasy Mini Kit was used to clean up the RNA by removing all the enzymes and similar entities, after which the RNA concentration was measured using the Nanodrop device.

Fluorescent *In situ* hybridisation (FISH)

Preparing of adrenal cryosections

Adrenal glands from adult Wistar-rat were used. Adrenal glands were collected as fast as possible after Euthanasia. Adrenal glands were cleaned and mounted with cryosection mounting media OCT (Cell path) in previously prepared moulds. The tissue sections were prepared using a cryostat (Bright 5040) to a thickness of 25µm and were placed onto new Superfrost plus or Polysine slides which are suitable for *in situ* hybridisation and immunohistochemistry. Each of these steps was carried out in RNAse-free conditions.

FISH

The followed procedure was previously described (Al-Bedhawi, 2018).The adrenal glands sections were thawed and dried then fixed with 4% (w/v) paraformaldehyde (PFA) in phosphate buffer saline (PBS) for 20-25 minutes. The sections were then washed with PBS, dehydrated using a series of ethanol solutions, samples were di-lipidated in chloroform and rehydrated using the opposite ethanol concentrations. The slides were washed with PBS before being treated with proteinase K (Roche) 100µg/ml in RNase free water for 30 minutes at 37°C in order to increase the permeability.

The pre-hybridisation step was conducted with a hybridisation buffer for 4 hours at 60°C to block non-specific binding. The sections were then exposed to hybridisation buffer containing the labelled probe (500ng/ml) of interest for 26-30 hours at 60°C in a sterilised and RNase-Zap treated humidified chamber. Post hybridisation steps were conducted using 5xSSC, RNA wash buffer and 0.1X SSC. A polyclonal sheep anti-digoxigenin (Fab-segments), conjugated to alkaline phosphatase (750 U/ml) (Roche,) was diluted 1:5000 (150 mU/ml) in a blocking buffer

The hybridisation signals were detected using the ELF 97 mRNA *in situ* hybridisation signal detection kit (Molecular Probes). The sections were mounted by applying 3–5 drops of mounting medium (ELF 97) to each slide and placing a coverslip on top and these slides were examined using a fluorescent microscope with the 546nm filter on 5x and 20x objective.

Immunohistofluorescence

Adrenal cryosections were prepared as previously described with the exception of the thickness of the sections, which was 10µm rather than 25µm. The slides were dried for 20-30 minutes at room temperature and then were fixed by immersion in a jar full of pre-cooled acetone for 20 minutes. After which they were removed and allowed to dry for 20-30 minutes at room temperature.

Slides were washed twice with phosphate-buffered saline containing PBS with Triton (PBT) for 5 minutes each and blocked with blocking buffer (10% v/v goat serum in PBS for 1 hour; the serum is derived from the species in which the secondary antibody was raised, usually goat serum). The primary antibody working solution was prepared by mixing the suitable amount of primary antibody with an antibody dilution buffer (0.5% v/v of the same serum in PBS). A drop (100-200µl) of the primary antibody working solution was applied to each section and they were incubated in a humidified chamber overnight at 4°C.

The sections were each washed twice with PBT for 5 minutes before the addition of a drop (100-200µl) of diluted (1:200) secondary antibody in the antibody dilution buffer. Then they were incubated in the dark, in a humidified chamber, at room temperature for 30 minutes before being washed twice in PBS for 5 minutes each. The sections were then mounted in mounting medium for fluorescence with DAPI (Vectashield) to stain the nucleus and covered with coverslips. The control sections were treated with the appropriate IgG protein instead of the primary antibody. In the dual immunohistofluorescence both primary and secondary antibodies (mouse and rabbit) were mixed together in the primary and secondary incubation steps, respectively.

Microscopy

The immunofluorescent imaging for *in situ* hybridisation and immunohistofluorescence experiments was carried out using a Zeiss-Axio Imager A1 fluorescent microscope with four filters: Blue/cyan filter for (4',6-

diamidino-2-phenylindole (DAPI)), Alexa Fluor (AF) 488 green, AF 546 yellow and AF 660 red. The magnification powers of the objective lenses used in these experiments were 5x, 10x, 20x and 40x. The fluorescent microscope was fitted with a camera (Axio cam MRm) linked to a computer and controlled by AxioVison software (Rel.4.8). This software was used to adjust the colours, measures, and quality of the images. The quality of the images was adjusted using the microscope fine adjustment. Image J software was used for co-expression analysis.

RESULTS AND DISCUSSION

Tmem121 was selected as a potential proliferation cell marker of the adrenocortical cells after a bioinformatics analysis of a whole rat adrenal transcriptome microarray data set. This data showed the expression of this marker based on its high expression in the capsule, or capsule and zI, in comparison with zF (table.2). The expression values in the microarray data were manipulated to be displayed in fold differences between the capsule, or capsule and zI, in comparison with zF. The selection of this potential marker was mainly based on a combination of the array data and the information in the literature that implied potential roles in stem/progenitor cells characteristics, cell proliferation of Tmem family members in general and specifically of Tmem121 as a rarely studied transmembrane protein.

Several molecular experiments were conducted in order to examine the expression of this marker followed by cloning a fragment of this gene into the p-GEM®-T easy vector. This was followed by *in vitro* transcription to produce probes to detect the mRNA expression patterns of this marker in the rat adrenal cortex by FISH. Immunohistofluorescence experiments also followed to detect the localisation of the protein of interest in adrenal sections.

Table 1: Microarray data show the expression levels of Tmem121gene in the capsule, zF and zI. The gene has high expression in the capsule and zI (the potential stem/progenitor cell layers) in comparison with zF.

Gene title	Gene symbol	Capsule	zF	zI
Transmembrane protein121	Tmem121	138.4809	2.175656	68.63601

Extraction of RNA from adrenal tissue

The extracted RNA was analysed using agarose gel electrophoresis (figure 1) and spectrophotometry. The first extracted RNA from one frozen adrenal was 125.3ng/µl and the A260/230 ratio was 2.19, which indicated low contamination with either polyphenol or polysaccharide. The A260/280 ratio was 1.8 which indicated very low protein or phenol contamination. As opposed to frozen adrenal glands that stored in -80 ºC for over six months, RNA extraction from freshly isolated adrenal gland showed high quality RNA (as it measured using a spectrophotometric method) and the concentration of RNA was 400-1200 ng/µl per one adrenal gland.

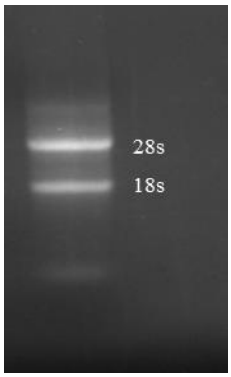

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Figure 1. Agarose gel electrophoresis of 10µl of 125.3 ng/µl RNA after isolation from whole rat adrenal using Trizol method. The appearance of 28S and 18S ribosomal RNA subunits bands indicate the RNA was obtained with minimum degradation.

First strand cDNA synthesis

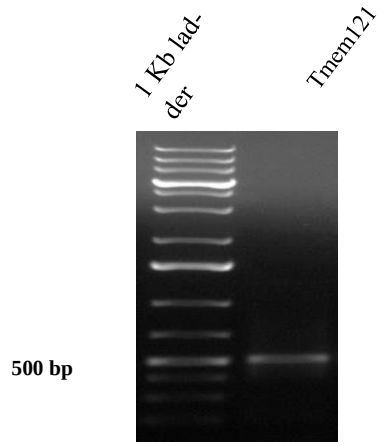
cDNA synthesis resulted in the generation of single stranded cDNA from the isolated RNA. Analysis by spectrophotometry showed that it had a concentration of 77ng/µl.

Gene detection by PCR and agarose gel electrophoresis

Polymerase chain reaction (PCR) was carried out to detect the mRNA expression of the Tmem121 in the adrenals and to provide template fragmetns suitable for riboprobe generation. The bands were excised from the gel and purified using the Qiagen QIAquick Gel Extraction Kit. The concentrations of these segments after purification were measured by spectrophotometry and showed to be 28-42ng/µl.

Transformation of *E.coli* with the cloned segments

The DNA segments were ligated into the p-GEM®-T Easy plasmid and subsequently transformed into competent *E. Coli* cells. The transformed colonies were grown on LB agar plates; the transformed colonies appeared after 10 hours of incubation while the negative control group showed no growth.



. **Figure 2 Agarose gel electrophoresis of 10µl of the PCR products that represent an approximatly 500bp segment of Tmem121. The PCR was conducted using specific primers to detect it from rat whole adrenal cDNA. This picture reveals the presence of the targeted band segments.**

Plasmid isolation and restriction enzyme digestion

In order to confirm the ligation of an insert into the vector, one colony was picked from each plate and grown in 10ml of LB broth. Plasmids were then isolated using a Qiagen miniprep kit and then subsequently digested with *EcoR1* in a total volume of 10µl. Since the restriction sites of *EcoR1* in the p-GEM®-T easy plasmid was adjacent to the two ends of the insert the isolated plasmids from the colonies were digested with *EcoR1* to confirm that the segments were successfully cloned. After digestion, the products were separated by agarose gel electrophoresis (Figure 3). The bands of each digested plasmid were observed, where the upper band represented the plasmid backbone (approximately 3000bp), and the lower bands were the inserts of approximately 500bp. The successful cloning was also confirmed by sequencing.

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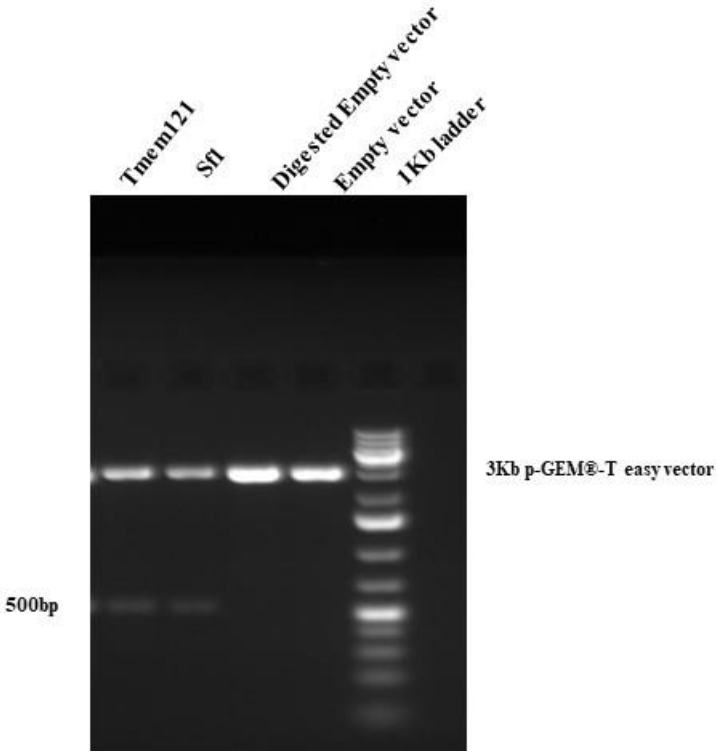

Figure 3 Agarose gel electrophoresis of the cloned inserts of Tmem121 with Sf1 insert as control and the mere-vector (p-GEM®-T easy), after and before digestion with *EcoR1*. The digestion releases the inserts from the vector, which were then separated by their size allowing profiling on an agarose gel. This analysis showed the appearance of digested DNA of the inserts at around 500bp, where the larger bands (3,000bp) represent the vector backbone

Generation of templates suitable for in vitro transcription

RNA-probes were not generated from the insert in the p-GEM®-T Easy vector by digestion with an appropriate restriction enzyme and then using either the T7 or SP6 RNA promoters to initiate transcription. However, amplification of the desired segment by PCR was preferable as this added either T7 or SP6 promoter to the insert and the DNA product of the PCR were much more than the DNA product from the digestion. To achieve this, PCR was used with one primer specific for the insert together with either an M13 forward or reverse primer. This revealed the orientation of the insert in the vector at the same time as adding either T7 or SP6 RNA polymerase promoter sequences to the insert. The results revealed that Tmem121 inserts was ligated into the vector in the correct orientation such that the T7 promoter sequence of the plasmid could be used to generate the sense RNA of the insert. In contrast, the Sf1 insert which used as a control insert was in the opposite orientation such the SP6 promoter sequence could be used to generate sense RNA of the insert (Figure 4).

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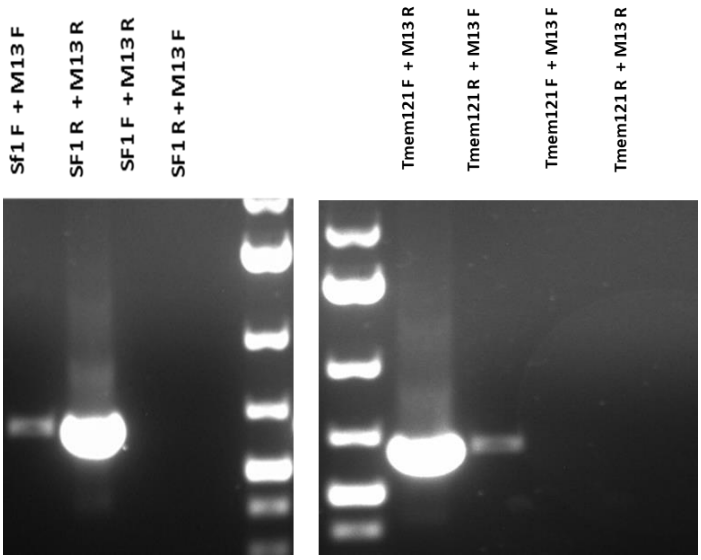

Figure 4. Agarose gel electrophoresis of the PCR products of the Tmem 121 and the control (Sf1) with a segment of the T7 or SP6 promoters that were added by using M13 primers in all possible combinations. This in turn referred to the direction in which the inserts had ligated to the vector. A showed the products of all similar primer combinations (insert F primers with M13F primers and insert R primers with M13 R primers). This showed the Sf1 bands, revealing its opposite orientation in the vector. B showed the products of all different primer combinations (insert F primers with M13R primers and insert R primers with M13F primers). This showed the bands of Tmem121 revealing its correct orientation in the vector.

***In vitro* transcription and RNA purification**

DIG labelled RNA probes were synthesised by *in vitro* transcription using the DNA segments described in section 3.7 along with the appropriate RNA polymerase. The RNA probes were purified using the RNeasy Mini Kit (Qiagen) and analysed using agarose electrophoresis (figure 5) to check that the synthesis was successful. The concentration of the Klf5, Thy-1, Tmem121 and SF1 probes was determined by spectrophotometry to be 1780ng/µl, 1903ng/µl, 1650ng/µl and 1245ng/µl, respectively. The concentration of the probes decreased dramatically after purification.

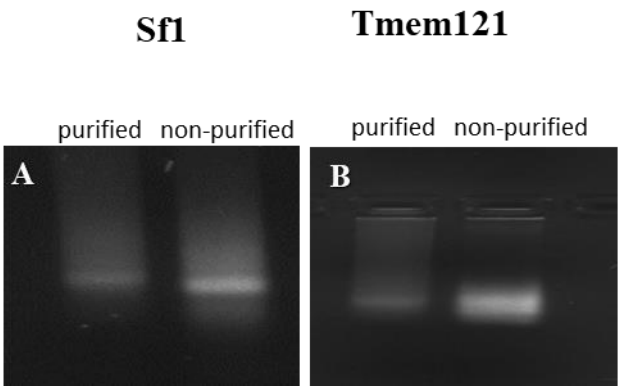

Figure 5. Agarose gel electrophoresis of the RNA probe bands of Tmem121 and Sf1 after *in vitro* transcription. (A) The antisense RNA probe bands of Sf1. (B) The antisense RNA probes bands of Tmem121. The right-hand bands shown in A and B are the probes before purification, while the left-hand bands are after purification.

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Fluorescent *in situ* hybridisation (FISH)

The mRNA expression of Tmem121 was detected in normal adult rat adrenal glands using fluorescent *in situ* hybridisation. Expression of Tmem121 was found mainly in the capsule and sub-capsular areas. Tmem121 expression was low in the zG and zF (definitive zones). The mRNA expression of Sf1 (NR5a1) in this study showed a typical Sf1 expression which expressed in most of the adrenal cortex (Figure 6).

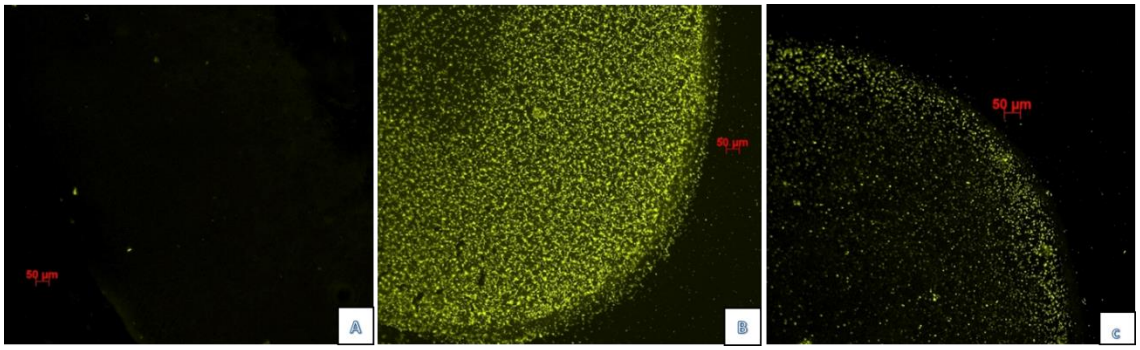

Figure 6. The expression areas of the targeted mRNA using Riboprobes on the adrenal sections (cryo-sectioning) by using *In situ* hybridisation technique. (A and B) are the control groups; (A) The negative control sections (no probes), (B) The first positive control sections (with the antisense NR5a1 probe). (C) The adrenal section (with the antisense Tmem121 probe).

Immunohistofluorescence

The detection of the mRNA of the gene of interest using FISH, was followed by detection of the protein expression of the Tmem121 using immunohistofluorescence because it was necessary to confirm that these transcripts were being translated.

Tmem121 proteins were detected in normal adult rat adrenal glands. Using primary antibody against Tmem121 protein and IgG protein as a control. In agreement with the *in situ* hybridisation results, the Tmem121 protein expression results were mainly observed in the subcapsular area. The pattern of Tmem121 protein expression was ranging from a few cells in the sub-capsular area to a long line of cells along the capsule with slight expansion toward the zG (Figure 7).

Due to the lack of information regarding Tmem121 and the pattern of its area of expression in the adrenal cortex, dual antibody detection experiments were conducted to assay the co-expression of Tmem121 with the proliferation marker Ki67 (Figure 8). The result of co-expression between the two proteins was 0.74 (according to Spearman's rank correlation value, which was calculated using Image J 1.49 software). This represents high (74%) but not complete co-expression of the Tmem121 with the proliferation marker Ki67.

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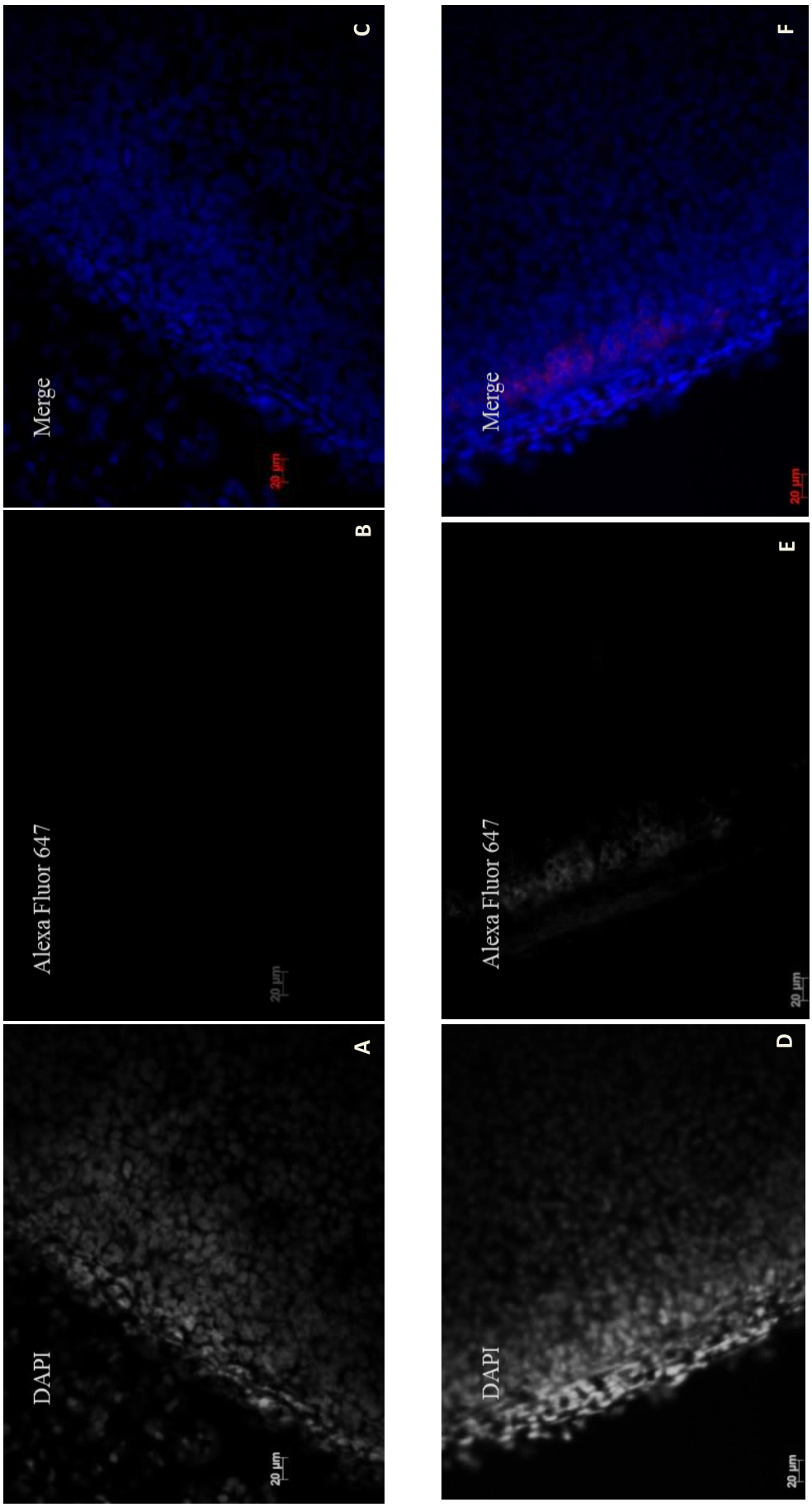

Figure 7. ***Immunohistofluorescence of*** **Acetone fixed cryosections of rat adrenal cortex capsular and sub capsular cells** ***using*** **anti-Tmem121 antibody (ab151077)to detect Tmem121 protein to detect. (A-C) Tmem121 negative cells. sections were incubated without the anti-Tmem121 antibody. (D-F) Tmem 121 positive cells. sections were incubated with the anti-Tmem121 antibody (ab151077). Cells expressing Tmem121 identified by the red fluorescence.**

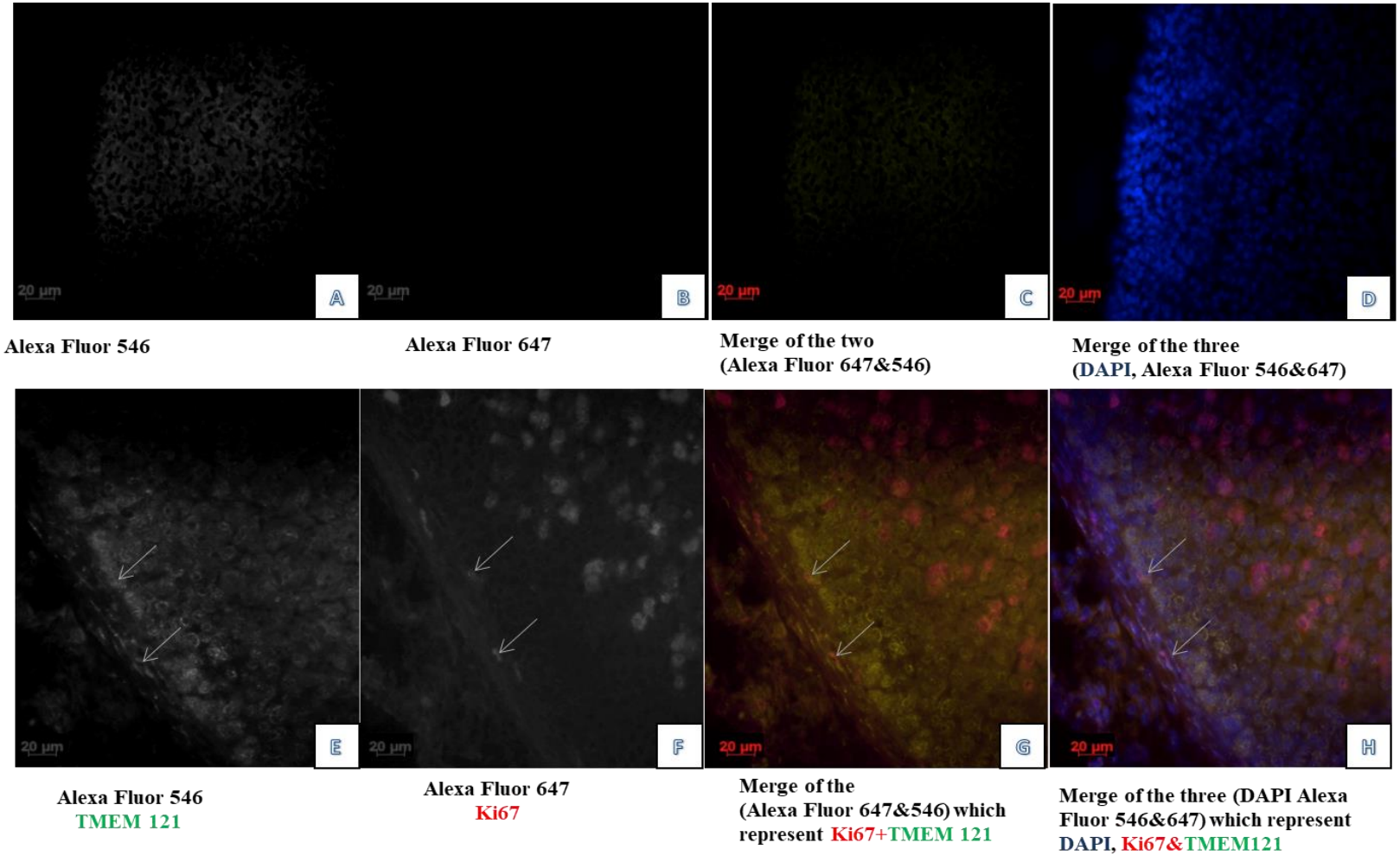

Figure 8: I*mmunohistofluorescence of* Acetone fixed cryo-sections of rat adrenal cortex measuring the co-localisation of TMEM 121(ab151077) with Ki67(BD Pharmingen550609) . (A-D) Tmem121 and Ki67 negative cells, sections were incubated without the anti-Tmem121 and Ki67 anti-bodies. (E-H) Tmem121 and Ki67 positive cells, sections were incubated with the anti-Tmem121 and Ki67 antibodies. Cells expressing Tmem 121 identified by the yellow fluorescence, Cells expressing Ki67 identified by red fluorescence. Arrows referred to the co-localisation spots.

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DISCUSSION

Several studies referred to the existence of stem/progenitor cell niche in the adrenal gland cortex were the cells undergo relatively high proliferation activity. Most of these studies were considered the capsule as stem cell niech (Walczak and Hammer, 2015) (mohammed 2018). Others proposed the zI in rodents such as rat (Mitani, 2014) as potential niches for adrenocortical stem/progenitor cells. However, the expression pattern of the Tmem121 marker was mainly beneath the capsule where most of proliferation activities occurring (Freedman et al., 2013).

Although the array data showed an interesting expression pattern in the capsule with low expression in the zF, few studies includedTmem121. Since Tmem family members are highly expressed in most embryonic tissues (Zhou et al., 2005), that might involve in the characteristic of stem/progenitor cells. The only related study to the Tmem121 is a human wound healing study that showed the relationship of Tmem121 proliferation and migration of human endothelial cells and its expression was co-expressed with the endothelial marker, CD31. Therefore, these results suggested that it might have a potential role in skin wound healing (Bronneke et al., 2015). The results of the FISH and the immunohistofluorescent assays of Tmem121 mRNA and protein expression showed an interesting pattern in the proliferating area of the adrenal cortex which supports the hypothesis of the Tmem121 role in the proliferation of adrenocortical cells. The dual immunohistofluorescent assay that showed the co-expression of the proliferation marker Ki67 with the Tmem121 showed a high but not full co-expression of both proteins. This un-completed co-expression might result from the expanded expression of Ki67 in the adrenal cortex (figure7) which involve in every cell division because this marker organises heterochromatin in the proliferating cells (Sobecki et al., 2016). while the expression of Tmem121 was mainly restricted to the capsule and sub-capsular areas (figure7). These findings suggested the a role of Tmem121 in the proliferation of adrenocortical stem/progenitor cells.

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