Towards the generation of adrenocortical cells from pluripotent stem cells

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ÉCOLE DOCTORALE
SCIENCES DE LA VIE ET DE LA SANTÉ

THÈSE DE DOCTORAT

Vers la génération de cellules corticosurrénales à partir de cellules souches pluripotentes

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Présentée en vue de l'obtention du grade de docteur en Sciences de la Vie et de la Santé **d'**Université Côte d'Azur
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Soutenue le : 17/12/2021

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IFCAH International Fund Congenital Adrenal Hyperplasia

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Résumé

La corticosurrénale (CS) est un organe central stéroïdogénique qui joue un rôle clé dans le maintien de l'homéostasie de l'organisme. Plusieurs maladies des surrénales pourraient en principe être réparées en corrigeant la mutation ou en introduisant un transgène portant une forme sauvage du gène muté pour restaurer de façon permanente l'activité enzymatique. Cependant, les données de notre groupe et d'autres groupes démontrent un renouvellement rapide du cortex surrénalien. Ainsi, les cellules stéroïdogéniques qui ont été génétiquement modifiées sont susceptibles d'être rapidement remplacées par amplification des progéniteurs pouvant encore porter la mutation. La correction génétique devra donc cibler les populations de cellules souches surrénaliennes (CSS) plutôt que les cellules stéroïdogéniques entièrement différenciées, et l'approche in vitro semble être la meilleure. En principe, deux voies alternatives pourraient être envisagées : 1) Génération de cellules pluripotentes induites (IPSC) à partir d'un patient, correction de la mutation à l'aide d'une approche CRISPR/Cas9 et différenciation ultérieure vers la lignée surrénalienne avec transplantation dans/sous la capsule surrénalienne du patient. 2) Isolement et culture de CSS, correction in vitro suivie d'une transplantation chez le patient. Le but de ce projet est d'établir un protocole pour la différenciation in vitro de cellules ES de souris (mESCs) en cellules progénitrices surrénaliennes et d'évaluer leur aptitude à être transplantées sous la capsule surrénalienne. Pour atteindre cet objectif, j'ai décidé de développer une procédure de différenciation par étapes qui suit autant que possible le développement normal. Le primordium adréno-gonadique (AGP) se développe à l'interface du mésoderme intermédiaire antérieur et du mésoderme latéral. J'ai développé un protocole robuste qui permet la différenciation in vitro de mESC via l'état EpiSC et l'état de ligne primitive, en mésoderme intermédiaire antérieur et latéral. Une différenciation correcte a été démontrée par l'expression de marqueurs spécifiques du type cellulaire, notamment Brachyury (T) pour la ligne primitive, Osr1, Gata4 et WT1 pour la lignée du mésoderme, LIM1 et PAX2 pour le mésoderme intermédiaire antérieur, et Foxf1 et Prrx1 pour le mésoderme latéral. Les voies de signalisation qui sous-tendent la spécification des organes stéroïdogéniques ne sont pas encore bien établies. Pour mieux comprendre ce processus, nous avons établi une collaboration avec le professeur Serge Nef (Université de Genève), dont le laboratoire a réalisé des expériences RNA-Seq sur cellules uniques à des moments clé de la différenciation adréno-gonadique et de la séparation du primordium adréno-gonadique (AGP) en primordium surrénalien (AP) et primordium gonadique (GP). En utilisant les informations extraites de cet atlas cellulaire, et en testant divers activateurs et inhibiteurs de voies, j'ai pu orienter davantage la différenciation vers le destin stéroïdogénique précoce, comme le démontre la régulation à la hausse de Nr5a1, un régulateur principal de la stéroïdogénèse. En testant une série de protéines de la matrice extracellulaire, j'ai pu montrer que la fibronectine 1 (FN1) augmentait la production de cellules positives NR5A1. De plus, l'induction de la voie PKA à l'aide d'un dérivé de l'AMPc (8-Br-cAMP) a d'avantage augmenté les niveaux d'expression de NR5A1 dans ces conditions, tant au niveau de l'ARN que de la protéine ; mais toujours en nombre limité pour revendiquer une grande efficacité de protocole. En outre, la culture des cellules en 3 dimensions a induit Nr5a1 et d'autres marqueurs précoces de progéniteurs surrénaliens par rapport aux marqueurs gonadiques dans des conditions spécifiques. Des recherches supplémentaires sur les aspects clés de la différenciation in vitro sont nécessaires pour établir un protocole robuste d'organoïdes surrénaliens. Enfin, j'ai pu partiellement transposer le protocole aux hIPSC, dans lesquelles les cellules étaient correctement destinées à la lignée des progéniteurs stéroïdogéniques précoces. L'ensemble de ces résultats fournit une feuille de route pour la différenciation des cellules souches pluripotentes en progéniteurs surrénaliens et constituera la base de futurs travaux sur les thérapies de transplantation des maladies surrénaliennes.
Mots-clés : Cortex surrénalien, Nr5a1, Développement, différenciation in vitro, ECM

Abstract

The adrenal cortex (AC) is a central steroidogenic organ with key functions in maintaining body homeostasis. Several adrenal diseases (eg Congenital adrenal hyperplasia (CAH)) could in principle be repaired by correcting the mutation (e.g. via recombination) or introduction of a transgene carrying a wildtype form of the mutated gene to permanently restore enzyme activity. However, data from our and other groups demonstrate a rapid turnover of the adrenal cortex. Thus, steroidogenic cells that have been genetically modified are likely to be rapidly replaced by amplifying progenitors that may still carry the mutation. Genetic correction will therefore need to target adrenal stem cell (ASC) populations rather than fully differentiated steroidogenic cells, and in vitro seems to be the better approach. In principle two alternative routes could be envisaged: 1) Generation of induced pluripotent cells (IPSC) from a patient, correction of the mutation using a CRISPR/Cas9 approach and subsequent differentiation towards the adrenal lineage with transplantation in/under the patient's adrenal capsule. 2) Isolation and culture of ASCs, correction in vitro followed by transplantation back into the patient. Aim of this project is to establish a protocol for the in vitro differentiation of mouse ES cells (mESCs) into adrenal progenitor cells and to evaluate their suitability for transplantations under the adrenal capsule. To achieve this goal, I decided to develop a stepwise differentiation procedure that follows as much as possible normal development. The adreno-gonadal primordium (AGP) develops at the interface of the anterior intermediate and lateral plate mesoderm. I developed a robust protocol that allows in vitro differentiation of mESCs via the EpiSC and primitive streak state, into the anterior intermediate and lateral plate mesoderm. Proper differentiation was demonstrated by the expression of cell type specific markers including Brachyury (T) for the primitive streak, Osr1, Gata4 and WT1 for mesodermal lineage, LIM1 and PAX2 for the anterior intermediate, and Foxf1 with Prrx1 for lateral plate mesoderm. The pathways underlying the specification of steroidogenic organs are not yet well established. To obtain further insight into this process, we established a collaboration with Prof. Serge Nef (University of Geneva), whose laboratory has performed single cell RNA-Seq experiments at critical time points of adreno-gonadal differentiation and separation of the adreno-gonadal primordium (AGP) into adrenal primordium (AP) and gonadal primordium (GP). Using information extracted from this cell atlas, and by testing various pathway activators and inhibitors, I was able to further orient differentiation towards the early steroidogenic fate as demonstrated by the upregulation of Nr5a1, a master regulator of steroidogenesis. By testing a range of extracellular matrix proteins, I could show that fibronectin 1 (FN1) enhanced the production of NR5A1 positive cells. Moreover, induction of the PKA pathway using a cAMP derivative (8-Br-cAMP) further increased NR5A1 expression levels in these conditions, both at the RNA and protein level; but still in limiting numbers to claim high efficiency of the protocol. In addition, culturing cells in 3D induced Nr5a1 and other early adrenal progenitor markers over gonadal ones in specific conditions. Further investigation for key aspects of the in vitro differentiation is needed to establish a robust adrenal organoid protocol. Finally, I could partially translate the protocol to hIPSCs, in which the cells were fated correctly for the early steroidogenic progenitor lineage. Taken together these results provide a road map for differentiation of pluripotent stem cells into adrenal progenitors and will form the basis for future work towards transplantation therapies of adrenal diseases.

Key words: Adrenal Cortex, Nr5a1, Development, in vitro differentiation, ECM

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1. Introduction

The adrenal gland is responsible of maintaining body homeostasis through regulation of various processes such as blood pressure, inflammation, stress and metabolism. The adrenal gland is one of the least studied organs of the human body, and it steadily shows that it is an organ affected greatly by our modern lifestyle. The adrenal glands were termed by their location in Latin, ad = near and renes = kidney, or suprarenal, where supra = above (Neville & O'Hare, 1982). Shortly after, a nerve connecting the adrenal gland was described, suggesting a neuroendocrine role for the adrenal gland (Leoutsakos & Leoutsakos, 2008). In the beginning there was a debate if there were an extension or part of the kidney, due to their localisation and close attachment to them, but anatomical studies proved their separate entity (Neville & O'Hare, 1982). Thomas Addison was the first to describe the phenotype and pathology of adrenal insufficiency, and the condition of adrenal insufficiency and its related clinical manifestations were named after him (Addison's disease) (Leoutsakos & Leoutsakos, 2008; Pearce, 2004). 10 years after the adrenal cortex and its cellular organization in the different concentric zones was further described. Cushing's Syndrome, which is a form of overactivation of the adrenal gland resulting in the pathology of hypercortisolism, was medically described and it was another milestone in adrenal research (Walczak & Hammer, 2015). Finally, after decades of research on adrenal extracts and *in vitro* analysis of its hormones, the Nobel Prize was awarded to Edward Calvin Kendall, Philip Hench and Tadeusz Reichstein for their discoveries and understanding on the adrenal gland(Richard B. Welbourn et al., 1991).

1.1.a. Adrenal cortex during development.

The adrenal glands are bilateral structures on top of the kidneys, with two main structural and developmental different compartments, the cortex and the medulla (**Figure.1**). The adrenal cortex can be first identified as a thickening of the coelomic epithelium, and presumably shares common origin with the gonadal progenitor cells which is termed adrenogonadal primordium (AGP), which is marked by the expression of NR5A1. The more dorsal-medial part will give rise to the future adrenal cortex and the more ventral one to the gonads (Hatano et al., 1996a). The subset of AGP cells with the highest expression of NR5A1 is responsible for the formation of the adrenal cortex is the adrenal primordium (AP) and it migrates ventrolateral of the dorsal aorta where it settles (Luo et al., 1994a). The AP will get encapsulated and will give rise to the fetal adrenal cortex, which is then subdivided to the fetal (FZ) and definitive zone (DZ) (Oikonomakos, Weerasinghe Arachchige, et al., 2021). In human, after encapsulation, the fetal adrenal cortex expands dramatically due to the hypertrophy of the FZ and the hyperplastic identity of the DZ (Coulter et al., 1996; Johannisson, 1968), where the fetal adrenal cortex is one of the largest organs and the highly steroidogenic FZ cells compose most of it. Primates' fetal adrenal cortex development and homeostasis can differ from the rest species (Hornsby, 1985; Jaffe et al., 1981; Johannisson, 1968; McClellan & Brenner, 1981; Winter, 1985). The cells between FZ and DZ have a mix of characteristics, this population has been termed as transitional zone (TZ) (McNutt & Jones, 1970; Mesiano et al., 1993). The zones of the fetal adrenal cortex have different maturation rates and they have distinct steroidogenical identity (Mesiano & Jaffe, 1997). Until the formation of these zones, proliferating cells can be found throughout the fetal cortex (Bland et al., 2004; Zubair et al., 2009), where in later stages they are consecrated at the periphery (Schulte et al., 2007). Interestingly, fetal adrenal cortex is highly vascularized and vascular endothelial growth factor (VEGF) can potentiate this vascularization (McClellan & Brenner, 1981; McNutt & Jones, 1970; Shifren et al.,

1998). Another potent angiogenic factor, which has also significant effect in adrenocortical proliferation is basic FGF (Basile & Holzwarth, 1994; Gospodarowicz et al., 1985; Hornsby & Gill, 1977; Schweigerer et al., 1987). Indeed, mouse studies has shown the importance of FGF signalling in adrenal development (Guasti et al., 2013), and studies on human *in vitro* cultured FZ and DZ cells showed bFGF and EGF promotes their proliferation, with greater effect on DZ cells (Crickard et al., 1981; Hornsby et al., 1983).

The development of the adrenal cortex is described in more detailed on the following review article.

1.1.a.i. *Review on development of Adrenal Cortex*

Developmental mechanisms of adrenal cortex formation and their links with adult progenitor populations

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Molecular and Cellular Endocrinology 524 (2021) 111172

https://doi.org/10.1016/j.mce.2021.111172

Available online 21 January 2021

Introduction

Adrenals are central rheostats of the mammalian body that produce hormones to control an entire panel of physiological parameters. Based on their distinct embryological origin, their different morphological characteristics, and highly distinct hormone production, adrenals are subdivided into the capsule, cortex and medulla. The centrally located medulla is derived from Schwann cell precursors (SCPs)(Furlan & Adameyko, 2018) and produces catecholamines (epinephrine, nor-epinephrine) that have an important function in preparing the body for physical activity upon stress (fight-or-flight response). Both the formation and maintenance of the medulla are very different from the adrenal cortex, and a detailed description of these processes would be beyond the scope of this review. The interested reader is referred to a recent review on this topic (Kastriti et al., 2020). Here we will instead focus on the mesoderm-derived adrenal cortex and capsule that are tightly linked in terms of development and tissue homeostasis. The adrenal cortex is the source of several types of steroid hormones that are produced by highly specialised cells, which are arranged in concentric rings. Mineralocorticoids that are involved in blood pressure control are produced in the outermost zona glomerulosa (zG), glucocorticoids that are important in stress- and immune response are synthetized in the zona fasciculata (zF) and androgens that induce adrenarche in humans and some higher primates are released from the zona reticularis (zR). The capsule on the other hand can be considered as a signalling center that plays an important role in adrenal zonation and tissue maintenance.

Cells are constantly exposed to intrinsic and extrinsic insults and organs have developed mechanisms to counteract long term damage and ensure proper functioning throughout life. In most cases this is achieved through the activation of stem or progenitor cell populations. Recent research has revealed that the adrenal cortex is subject to very active cell renewal and several different adult progenitor cell populations have been identified. The purpose of the present review is to highlight these advances and relate them to cell populations and molecular mechanisms identified in development. As most of the studies have been performed in mice, descriptions below will refer to murine data, if not otherwise stated.

Developmental origins of fetal adrenocortical cells

Tissue renewal via stem/progenitor cells often employs mechanisms that are also found during development, and it is therefore important to consider how the adrenal cortex forms. Steroidogenic cells of the gonad and adrenal derive from a common adrenogonadal primordium (AGP) that can be identified by the expression of steroidogenic factor 1 (SF-1/NR5A1/ADB4), a master regulator of steroidogenesis (Luo et al., 1994b). (Luo et al., 1994; Sadovsky et al., 1995). Expression of *Nr5a1* has been reported as early as E9, a time point well before the AGP can be morphologically distinguished. *Nr5a1* RNA expressing cells extend from the coelomic epithelia of the urogenital ridge to the dorsal aorta. Based on their morphology, it is believed that those cells originate from the coelomic epithelium (CE) (Ikeda et al., 1994; K. I. Morohashi, 1997). The first cluster of cells expressing NR5A1 protein can be detected at E9.5-9.75 in the mouse (Bandiera et al., 2013; Fujimoto et al., 2013) and can be traced as a common primordium until E11.5. The molecular reason for the discrepancy between RNA and protein detection is presently unknown, but it may suggest that posttranscriptional mechanisms are in place to control NR5A1 protein production and/or stability. Subsequently, the AGP separates into the gonadal primordium (GP) located close to the coelomic epithelia, and into the adrenocortical primordium (AP) that is positioned closer to the dorsal aorta (Hatano et al., 1996b). Based on morphological studies and marker gene expression, mesoderm can be divided into paraxial (PM), intermediate (IM) and lateral plate mesoderm (LPM) (Figure 1). It has been debated whether the adrenal cortex originates from the LPM or the IM. Anatomical studies in sheep dating back to 1982 suggested a mesonephric (IM-derived) contribution towards the adrenal cortex (Upadhyay & Zamboni, 1982). However, knockout mice for *Pax2* or *Pax2*/*Pax8*, that are considered as markers of the IM, and *Emx2* showed normal adrenal development despite severe disruption of other organs that originate from the IM (Bouchard et al., 2002; N. Miyamoto et al., 1997; Pellegrini et al., 1997; Torres et al., 1995).

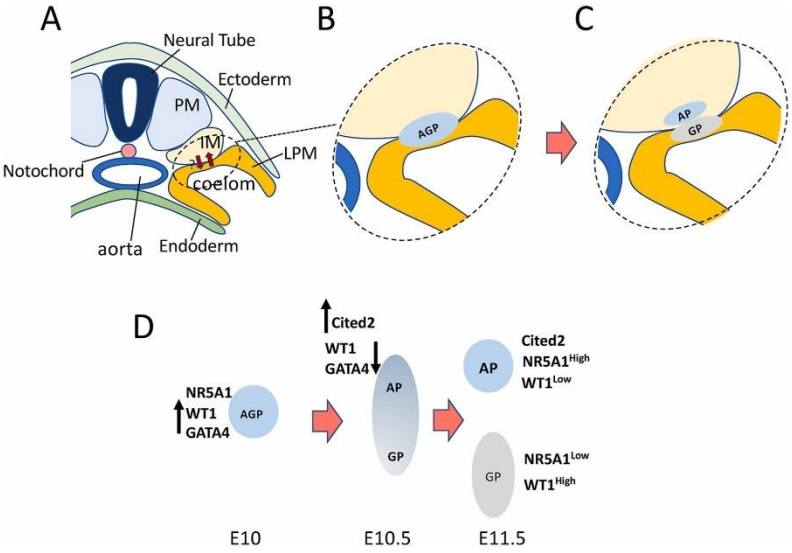

Fig. 1. Adrenal primordium specification. A) At early stages, the mesoderm can be divided into paraxial mesoderm (PM), intermediate mesoderm (IM) and lateral plate mesoderm (LPM). B) The adrenal gonadal primordium (AGP) is formed at the interface of the IM and LPM, probably through interactions between these two tissues. C) Soon after its specification, the AGP splits into adrenal primordium (AP) and gonadal primordium (GP). D) Key molecular markers during AGP specification and separation into AP and GP.

More evidence points to a LPM origin of the adrenal cortex. The first NR5A1-positive cells are found in, or close to the coelomic epithelium, a LPM cell layer covering the newly formed coelomic cavity. Most of the knockout studies in mice that affect LPM formation, cause severe heart and/or vascular defects and consequently result in premature death at around E9.5, a time point too early to evaluate

AGP formation (Ariza et al., 2016a; Heikinheimo et al., 1994; Ishitobi et al., 2011). However, from lineage tracing analysis using a *Prx1-Cre* line, in which Cre-recombinase is driven in the postcranial somatic lateral plate mesoderm, it can be observed that at least a proportion of adrenocortical cells have LPM origin (Durland et al., 2008; Kobus et al., 2015; Logan et al., 2002). Gonads, however, were not labelled in these experiments, a puzzling result when considering the model of a common AGP. Further analysis will need to be performed to resolve this enigma

The hypothesis of the LPM as the source for adrenal and gonadal somatic cells has also been supported by studies in chicken. Lineage tracing experiments suggest that GP-fated cells reside in the outer most layer of the CE, whereas cells that will develop into the adrenal cortex are situated in deeper layers (Funayama et al., 1999; Saito et al., 2017). To what extent this model is valid for mammalian adrenal development remains to be seen, in particular since avian and mammalian urogenital development differs in several aspects (Estermann et al., 2020; Sekido & Lovell-Badge, 2007).

In summary, published data would argue that the AGP, and by extension, also the fetal adrenal cortex originate from the LPM. We know however that mesonephric (IM-derived) cells actively contribute to gonad formation (Buehr et al., 1993; Capel et al., 1999; DeFalco et al., 2011; Tilmann & Capel, 1999), and an analogous process may also occur in the AP. Whether AP and GP are developing as independent populations or whether they truly share a common primordium also remains to be proven. Future lineage reconstructions using detailed single cell sequencing analysis may provide this answer.

Transcriptional control of Nr5a1

NR5A1 is a gene of paramount importance for steroidogenic organ development and absence of *Nr5a1* in mice leads to agenesis of adrenals and gonads and postnatal death of mutant animals (Luo et al., 1994b; Sadovsky et al., 1995). Since NR5A1 expression is the defining factor of the AGP, it is worth considering how its expression is activated and maintained. Regulation of the *Nr5a1* gene is complex and involves an initial activation in the AGP followed by the engagement of a fetal adrenal enhancer (FAdE) that is situated in intron 4 and is sufficient to drive high levels of expression in the fetal, but not in the adult adrenal cortex (M. A. Wood et al., 2013a; Zubair et al., 2006b). In the adult adrenal, *Nr5a1* expression appears to involve an autoregulatory feedback loop.

A key factor initiating *Nr5a1* transcription in the AGP is the Wilms' tumor suppressor WT1, a developmental regulator that is expressed in the nephrogenic cord and the overlying coelomic epithelium (Armstrong et al., 1993; A. W. Moore et al., 1998). Consistent with its essential role in regulating *Nr5a1*, genetic deletion of WT1 leads to loss of its expression and gonadal and adrenal agenesis (Bandiera et al., 2013; Kreidberg et al., 1993; Vidal & Schedl, 2000; Wilhelm & Englert, 2002). GATA4 and GATA6 have also a critical role for *Nr5a1* activation and are necessary for the early differentiation of steroidogenic cells in both AP and GP (Heikinheimo et al., 1994; Kiiveri et al., 2002). GATA4 function can be traced back to the CE of E8.75 mouse, and timed deletion interferes with AGP formation, as indicated by the lack of *Nr5a1* expression (Y. C. Hu et al., 2013). GATA4 has been shown to physically interact with WT1 and regulate the expression of *Sry* in gonads (Y. Miyamoto et al., 2008) and we can speculate that a similar interaction activates the *Nr5a1* promoter in the AGP, although this has not yet been demonstrated. When the AP has separated, GATA4 expression declines and GATA6 becomes more prevalent (Kiiveri et al., 2002; Padua et al., 2015; Tevosian et al., 2015). *Gata6* knockout animals develop adrenals, but zonation, homeostasis and steroidogenesis are disrupted. Furthermore, in the same animal model, patches of cells in the adrenal expressed gonadal markers, which may indicate a role for GATA6 in AGP separation or specification of the two primordia (Kiiveri et al., 2002; Pihlajoki et al., 2013). Activation of *Nr5a1* in the AP by WT1 seems to also involve recruitment of the transcriptional co-activator CITED2. Loss of CITED2 expression resulted in smaller adrenals that are positioned closer to the dorsal aorta, whereas the gonads did not show any obvious phenotype (Val

et al., 2007). Strikingly, despite its important role in *Nr5a1* activation in the AGP, WT1 needs to be repressed in the AP to allow activation of the steroidogenic program (Bandiera et al., 2013).

There is a number of other factors that appear to be important for *Nr5a1* expression. Genetic studies have demonstrated SIX1 and SIX4 as early regulators, and double mutants have lower NR5A1 levels and display smaller adrenals and gonads (Fujimoto et al., 2013; Kobayashi et al., 2007). M33, a polycomb related protein, is also required for full activation of *Nr5a1*, potentially by allowing chromatin opening at its locus (Katoh-Fukui et al., 2005). As mentioned above, strong expression of *Nr5a1* in the AP further involves the fetal adrenal enhancer (FAdE) situated in intron 4 of the *Nr5a1* gene. The FAdE contains PBX-HOX and PBX-PREP binding sites that are essential for its function before *Nr5a1* expression becomes autoregulatory (Zubair et al., 2006b). Consistent with the presence of PBX binding sites, *Pbx1* is expressed in adrenal precursors cells, and its deletion leads to the absence of adrenals (Schnabel et al., 2003; Zubair et al., 2006b). Finally, DAX1, a gene that is mutated in patients suffering from congenital adrenal hypoplasia and hypogonadotropic hypogonadism (Muscatelli et al., 1994; Zanaria et al., 1994), appears to regulate *Nr5a1* expression. Its precise role , however, is less clear as *Dax1* mutant mice show larger rather than smaller adrenals at birth, with a decline of adrenal tissue during adulthood(Scheys et al., 2011). On a molecular level DAX1 has been shown to suppress *Nr5a1* expression in the gonadal primordium. It also binds to FAdE, and its deletion leads to prolonged activity of this enhancer (Xing et al., 2017).

Taken together, we can identify several stages for fetal adrenal development (Figure 1D). The initial progenitor population at E9 mainly expresses WT1, GATA4 and possibly *Nr5a1* on the mRNA level. By E9.75, few cells in the urogenital ridge start to express NR5A1 protein, the hallmark of the AGP. At E10, cells that are fated to become AP cells activate *Cited2* (Val et al., 2007). During the separation (around E10.5), the AP population loses WT1 expression and increases NR5A1, whereas in the GP WT1 and NR5A1 are maintained at low levels. Of note steroidogenic Leydig or Theca cells also lose WT1, suggesting WT1 expression to be incompatible with steroidogenesis (M. Chen et al., 2017; Meng et al., 2019; L. Zhang et al., 2015).

Formation and zonation of the adult adrenal gland

Once the AP has formed, Schwann cell precursors migrate along peripheral nerves into the fetal adrenal to form the medullary chromaffin cells (Furlan et al., 2017) a process that appears to involve BMP signalling from the dorsal aorta (Saito & Takahashi, 2015) (Figure 2B). Although the fetal adrenal cortex is not crucial for the specification and localization of the chromaffin cells, it is paramount for the creation of the adrenal medullary tissue (Bland et al., 2004; Gut et al., 2005; Tevosian et al., 2015). The fetal adrenal cortex, on the other hand, can develop normally in the absence of the medulla, but the tissue becomes disorganized as development progresses (Britsch et al., 1998).

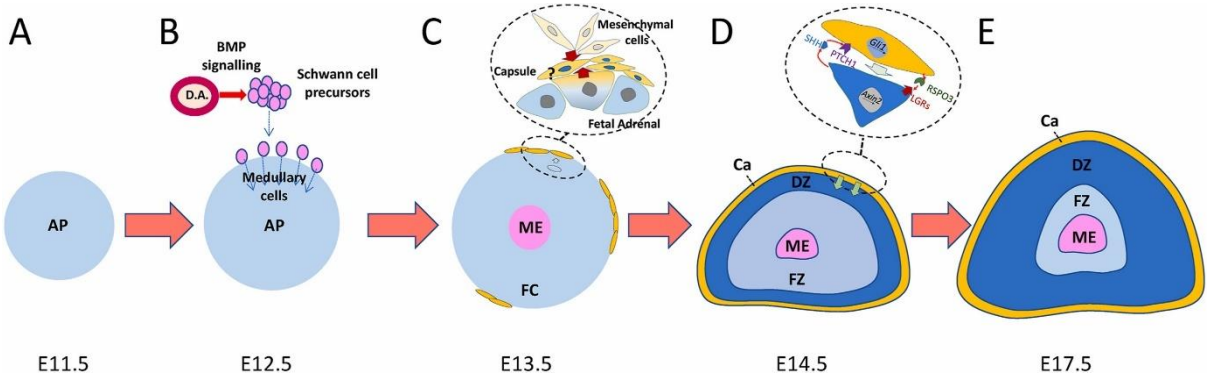

Fig. 2. Adrenal development. A) After separation of the AGP into AP and GP, (B) neural crest derived cells migrate into the AP to form the medulla, a process that involves BMP signalling from the aorta. C) Formation of the capsule involves the condensation of mesenchymal cells around the fetal cortex (FC). In addition, lineage tracing data suggest that cells from the

FC lose NR5A1 expression and contribute to the adrenal capsule. D) The adult adrenal cortex forms through recruitment of Gli1+ capsular cells (Ca) to the future definitive zone (DZ) and relies ona double paracrine mechanism involving the RSPO3/β-catenin and SHH signalling pathways (see inset). E) The definitive zone (DZ) further proliferates to replace the fetal zone (FZ), which however persists until puberty in males and the first pregnancy in females.

Whereas the AP gives rise to the fetal adrenal cortex, the adrenal capsule has been shown to contribute to the development of the definitive (adult) cortex (King et al., 2009). The precise developmental origin and composition of the capsule is, however, not yet completely resolved. Morphological and immunofluorescent studies have shown that at E12.5, mesenchymal cells that are positive for the transcription factors TCF21 and WT1 begin to condense around the AP to form a loose mesh of elongated cells (Bandiera et al., 2013)(Figure 2C). Since at this time the AP is embedded at the top of the nephrogenic cord, it seems likely that these cells are IM-derived. However, in lineage tracing experiments with a *Pax2:Cre* BAC line no adrenal labelling was reported (Ohyama & Groves, 2004). Lineage tracing experiments, however, suggest that early fetal adrenocortical cells also contribute to capsule formation. Indeed, in mice transgenic for a Cre^{ERT} transgene that is driven by the fetal adrenal enhancer of *Nr5a1* (*FAdE-*Cre^{ERT}*, Rosa26:lacZ*), a proportion of capsular cells becomes labelled when the CRE activity is induced at E11-E12.5. Lineage analysis demonstrates that these labelled cells are recruited during later stages of development and contribute to the formation of the definitive zone (DZ) (M. A. Wood et al., 2013a; Zubair et al., 2008b). These findings are compatible with a model in which a proportion of AP cells lose their NR5A1 expression and are maintained as steroidogenic progenitors in (or below) the capsule, to serve later as a reservoir for the formation of the adult adrenal cortex. However, we cannot exclude the possibility that the FAdE enhancer shows some ectopic activity in aggregating mesenchymal cells during capsule formation. Indeed, lineage tracing with a SF1:Cre line encoded on a much larger BAC construct did not show labelling in capsular cells (Bingham et al., 2006). Further experiments are therefore needed to determine the precise origin of steroidogenic progenitors of the adult adrenal cortex.

Once the capsule has formed, a double-paracrine mechanism is employed to recruit capsular cells to the steroidogenic lineage. RSPO3, a positive modulator of WNT/β-catenin signalling, is released from capsular cells to permit β-catenin signalling in the underlying fetal cortex (Vidal et al., 2016)(Figure 2D). One of the activated genes encodes the signalling molecule SHH, which in turn signals back to capsular cells, causing them to detach, migrate centripetally and differentiate into steroidogenic cells (King et al., 2009). Consistent with the important role of SHH signalling in adrenal development, steroidogenic cell specific (*Nr5a1:Cre* driven) deletion of *Shh* (Ching & Vilain, 2009; King et al., 2009) or truncation mutations in the SHH effector *Gli3* that mimic mutations found in Pallister – Hall syndrome, display severe adrenal defects (Grotewold et al., 2002; Hui & Joyner, 1993).

Growth of the DZ is not only fuelled by the recruitment of capsular cells, but also involves proliferation of NR5A1 positive cells. Several molecular pathways have been identified to drive this process. As described above, WNT/β-catenin signalling is active at a very early stage in adrenal development and is essential for DZ expansion, as demonstrated by a lack of adrenal cortex development in *Nr5a1:Cre,* $Ctnnb1^{flox/flox}$ animals (A. C. Kim et al., 2008). Several *Wnt* genes are expressed in the capsule and the underlying sub-capsular cells (Berthon et al., 2012), but to date no single knockout shows adrenal agenesis, which may suggest functional redundancy between WNT family members. By contrast, deletion of *Rspo3* leads to a complete loss of proliferation in steroidogenic cells and dramatically smaller adrenals (Vidal et al., 2016). RSPO3 acts by binding and inhibiting ZNRF3, an E3-ubiquitin ligase that suppresses WNT/β-catenin activity by sequestering the WNT receptor complex (Raslan & Yoon, 2019). Consistent with this suppressive role, deletion of *Znrf3* leads to increased cortical proliferation and adrenal hyperplasia (Basham et al., 2019).

FGF signalling is another important pathway that is involved in DZ expansion. Steroidogenic *Fgf1* and capsular *Fgf2* and *Fgf9* expression has been detected in E15.5 adrenals (Guasti et al., 2013). While the role of these signalling molecules in driving adrenal proliferation has not yet been genetically proven,

deletion of the isoform IIIb of the FGF receptor FGFR2 results in hypoplastic adrenals (Guasti et al., 2013). In parallel to WNT and FGF, Insulin/IGF signalling is essential for adrenal development. Simultaneous deletion of insulin receptor (INSR) and the IGF type I receptor (IGF1R) in mouse embryos leads to downregulation of NR5A1 expression and adrenal agenesis (Neirijnck et al., 2018; Pitetti et al., 2013). *In vitro* experiments suggest that insulin and IGFs act independently of the PKA pathway to activate *Nr5a1* expression and steroidogenesis respectively (Fottner et al., 1998; Kinyua et al., 2018).

The cell recruitment of capsular cells and proliferation of newly formed steroidogenic cells leads to the formation of a definitive zone (DZ) and concomitantly the displacement of fetal steroidogenic cells (also called fetal zone = FZ) towards the medulla (Figure 2E). In rodents, the FZ persist into adulthood as the X-zone but degenerates during pubertal maturity in males and after the first pregnancy in females (P. v Holmes & Dickson, 1971; K. ichirou Morohashi & Zubair, 2011; Zubair et al., 2006b) .

If all adrenocortical cells are derived from the same progenitors, how are the different hormone producing zones setup and maintained? Research over the last years have revealed that the antagonistic action between WNT and PKA signalling are central in controlling zonation (Drelon et al., 2016; Vidal et al., 2016; Walczak et al., 2014). As described above, RSPO3 released from capsular cells enhances WNT/β-catenin signalling, which activates the mineralocorticoid program and establishes the zG phenotype in subcapsular steroidogenic cells (Vidal et al., 2016). *Wnt4*, a target of β-catenin signalling in subcapsular steroidogenic cells, has been shown to reinforce the WNT/β-catenin signal (Dumontet et al., 2018). Consistent with this model, deletion of *Wnt4* leads to loss of mineralocorticoid production in the adrenal cortex (see below)(Heikkilä et al., 2002), whereas activation of β-catenin causes an expansion of the zG (Berthon et al., 2010; Pignatti et al., 2020). Recent data suggest that the formation of rosette structures, a hallmark of the zG, is orchestrated by β-catenin in two ways: First, it is likely to serve as a structural component of the adherens junction complex that is required for rosette formation although formal proof for this role is still missing. Second, canonical signalling induces genes including *Fgfr2*, which itself appears to be important for rosette formation and aldosterone production (Leng et al., 2020).

The continuous generation of new cells in the outer cortex leads to the centripetal displacement of cells, which as a consequence no longer receive WNT/ β-catenin activating signals, such as *Rspo3* (Vidal et al., 2016). The loss of this stimulus permits the activation of the PKA pathway that suppresses β-catenin activity and promotes the activation of zF specific genes and glucocorticoid production (Drelon et al., 2016). Deletion of EZH2, an essential component of the PRC2 complex that is involved in setting up repressive epigenetic marks on histones, appears to interfere with efficient zG to zF conversion (Dörner et al., 2017; Mathieu et al., 2018). The mechanisms driving differentiation of steroidogenic cells into cells of the zR are less understood, as mice do not have this zone. However, overactivation of the PKA signalling pathway through the deletion of its regulatory component R1α (*Prkar1a*$^{-/-}$) induces a zR-like phenotype in steroidogenic cells situated close to the medulla (Dumontet et al., 2018). It is therefore tempting to speculate that also in human, differentiation into zR cells is induced by high levels of PKA signalling.

Steroid progenitors in adult adrenal homeostasis

The adrenal cortex has a remarkable capacity to respond to mitogenic stimuli (Estivariz et al., 1982). After unilateral adrenalectomy in mice, the cortex of the contralateral adrenal displays compensatory growth with cell proliferation in the subcapsular zone resulting in cellular hypertrophy and hyperplasia (Beuschlein et al., 2002) . Moreover, studies performed almost 80 years ago in adult adrenalectomized rats, demonstrated that enucleated adrenal glands have the capacity to regenerate the organ (Baker and Baillif, 1939; Ingle and Higgins, 1938; Nabishah et al., 1998). The newly formed gland was functional and able to secrete corticosterone, indicating that the capsule or subcapsular tissue must contain stem/progenitor populations (Nabishah et al., 1998).

The adrenal cortex does not only respond to insults, but also undergoes rapid tissue renewal under homeostatic conditions with the entire cortex being replaced every few months (see chapter on sexual dimorphism for more details)(Grabek et al., 2019). At present we can only speculate about the necessity for this high cellular turnover. Steroid hormone synthesis generates toxic by-products such as isocaproic aldehyde, derived from cholesterol metabolism, and reactive oxygen species (ROS) (Prasad & Dhar, 2014). As these compounds may cause severe cellular damage, a continuous replacement of the cortex may be essential to ensure organ maintenance.

Historically, two alternative models for cortical tissue renewal have been proposed. 1) A zonal model, in which each zone is independent and can renew through zone-specific stem/progenitor cells (Deane and Greep, 1946; King et al., 2009). 2) A model with a common stem/progenitor cell that gives rise to all steroidogenic cell types (Gottschau, 1883; A. C. Kim et al., 2009; Nussmann. & Zwemer, 1941). In support of the latter model, the adrenal cortex show higher cell proliferation in capsular, sub capsular and outer cortex region, whereas higher cell apoptosis occurs at the cortico-medullary boundary in both rats (McNicol & Duffy, 1987; Zajicek et al., 1986) and mice (Grabek et al., 2019; A. C. Kim & Hammer, 2007). Using transgenic mice and chimeric rat models, further suggested that maintenance of the gland occurred from the capsular/subcapsular region toward the medulla (M. C. Hu et al., 1999; Iannaccone & Weinberg, 1987; Morley et al., 1996). Another proof for a common progenitor cell population for steroidogenic cells came from cell lineage tracing experiments with a zG specific aldosterone-synthase Cre line (*As:Cre*)(Freedman et al., 2013). These studies demonstrated that also in adults zG cells migrate towards the medulla undergoing lineage conversion along their migration path (Figure 2). Careful analysis has demonstrated rare cells positive for both CYP11B1 and CYP11B2 at the zG/zF boundary, which may represent a transient state for this conversion. Lineage conversion also occurs in the regenerating adult adrenal cortex after treatment with very high doses of dexamethasone (Freedman et al., 2013), a drug that supresses zF cells in mice through a negative feedback loop on the HPA axis (Grossmann et al., 2004). zG specific *As:Cre*-dependent deletion of *Nr5a1* prevented this transdifferentiation, but mice maintained a fully functional zF over several months, suggesting the existence of mechanisms to maintain the zF fate without prior differentiation into AS producing zG cells(Freedman et al., 2013). A potential explanation for this observation could be increased proliferation of already differentiated zF cells, or direct differentiation of stem cell populations into zF cells.

Whereas the model of centripetal migration appears to be straight forward, the cellular dynamics underlying cell renewal are complex and several cell populations that have progenitor cell characteristics have been identified (Figure 3). 1) The majority of renewal under homeostatic conditions seems to be ensured via *Shh* positive cells situated in the zG (King et al., 2009), that are NR5A1 positive and respond to WNT signalling, but are mostly negative for the steroidogenic enzymes *Cyp11B1* and *Cyp11B2* (Finco et al., 2018; Walczak et al., 2014). 2) *Gli1* positive capsular cells are a second well defined progenitor population that maintain their differentiation potential in adult adrenals (King et al., 2009). Whereas in male mice their contribution under homeostatic conditions is minimal (Finco et al., 2018; Grabek et al., 2019), they do contribute to steroidogenic cell renewal in females. Under challenging conditions, such as upon ablation of zF cells, recruitment increases (Finco et al., 2018). Capsular cells can therefore be considered as a reserve stem cell population. 3) *Tcf21*$^{+}$ progenitors that also reside in the capsule, have been shown to produce steroidogenic cells during early embryonic development. By contrast, in the adult adrenal cortex, this population only generates stromal cells including desmin-positive smooth muscle cells (SMC), and PDGFRA positive fibroblastic cells (M. A. Wood et al., 2013a). 4) WT1^{+} cells residing in the mesothelial lining of the adrenal capsule have also been reported to maintain steroidogenic potential (Figure 3B). Under normal conditions, activation of WT1^{+} mesothelial cells is rare, but when it occurs, cells proliferate, form networks of spindle shaped cells that gradually invade the steroidogenic compartment (Bandiera et al., 2013). These cells are characterised by the expression of WT1, GATA4 and low levels of NR5A1, a molecular profile that is reminiscent of early AGP progenitors. Hormonal changes, such as induced by

gonadectomy, leads to expansion and differentiation of WT1$^+$ cells that adopt gonadal and adrenal steroidogenic phenotypes, an observation that is in line with the model of a common AGP (Bandiera et al., 2013). 5) Finally, Nestin$^+$ cells that are situated close to nerve fibres within the subcapsular area and outer cortex appear (de Celis et al., 2015) to have the potential of differentiating into steroidogenic cells (Steenblock, Rubin de Celis, et al., 2018) (Figure 3C). The contribution of this cell type to steroidogenic lineage *in vivo* will require further confirmation and quantitative analysis, but it should be noted that *in vitro* Nestin$^+$ cells can be induced to produce steroid hormones (Steenblock, Rubin de Celis, et al., 2018).

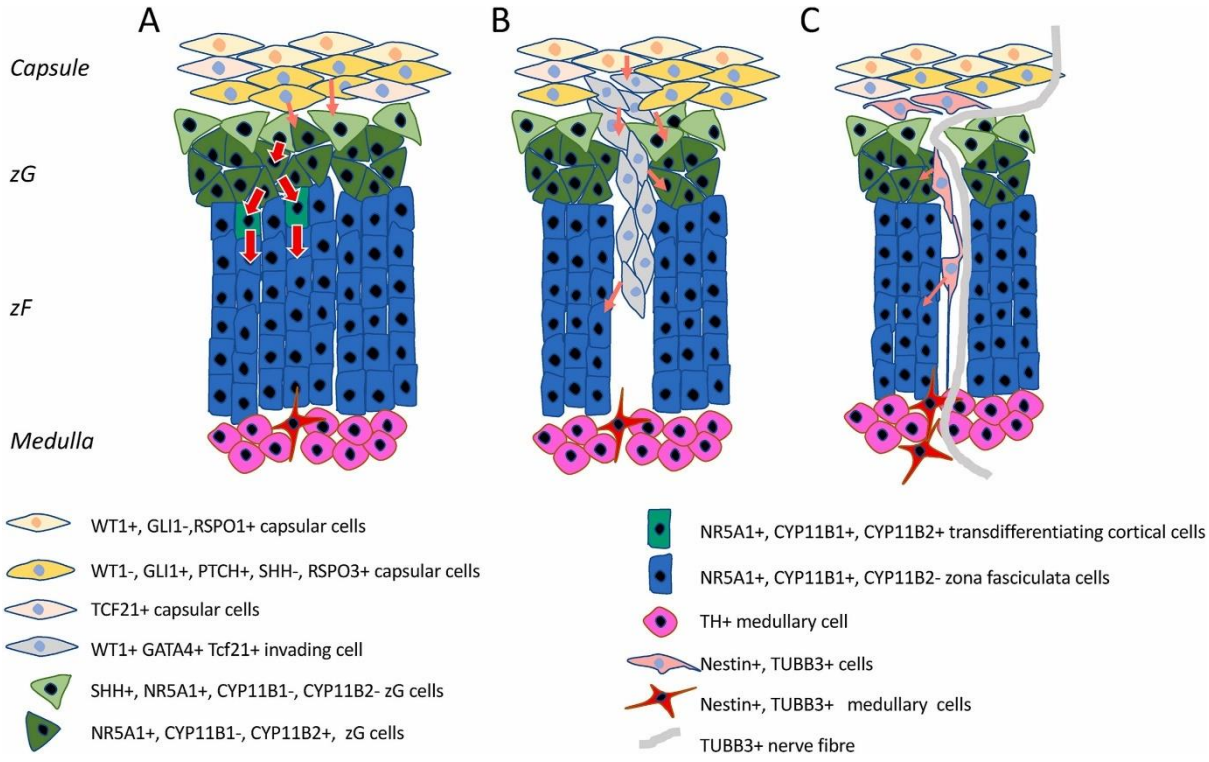

Fig. 3. Progenitor populations of the adult adrenal cortex. A) Under homeostatic conditions, maintenance of the adrenal cortex is ensured by Shh+ NR5A1+ positive progenitors within the zG (light green). To a lesser extent Gli1+ positive capsular cells are recruited in females, a process that is suppressed by androgens. B) In older animals and upon hormonal stress (gonadectomy) WT1+ cells lining the adrenal capsule can become activated to express GATA4/WT1/NR5A1low, invade the adrenal and further differentiate into steroidogenic cells. C) Rare Nestin+ cells in the outer cortex have also been reported to differentiate into steroidogenic cells in particular after repeated stress.

While the model of centripetal migration and lineage conversion fits well the data from mice, there is evidence that this mechanism may not be valid for all species. Studies in certain rat strains showed that there is an undifferentiated zone (uZ) of adrenal cells at the boundary between the zG and zF that do not produce steroids (Mitani et al., 2003). uZ cells can proliferate and migrate either centrifugally to form zG cells or centripetally to differentiate into zF cells (Mitani et al., 2003; Wright & Voncina, 1977). Similarly to progenitors in the zG in mice, uZ cells express *Shh* (Guasti et al., 2011).

Under normal conditions, differentiation of progenitors into steroidogenic cells is tightly regulated and considered unidirectional. However, in ageing mouse adrenals, progenitor cells from the adrenal capsule can accumulate and give rise to GATA4 and WT1-positive cells (Bandiera et al., 2013; Dörner et al., 2017; Mathieu et al., 2018a). In addition, loss of EZH2 appears to induce dedifferentiation of steroidogenic cells into progenitor-like cells expressing GATA4 and WT1 (Mathieu et al., 2018a). To what extent this accumulation of progenitor-like cells contributes to disease is presently unclear, but it has been speculated that GATA4/WT1 positive cells contribute to the development of adrenocortical tumours (Bielinska et al., 2009).

Sexual dimorphism in adrenocortical cell renewal

A large number of adrenal diseases show a strong sex bias with women being disproportionally frequently affected (Audenet et al., 2013a; Lacroix et al., 2015; Levasseur et al., 2019; Nishijima David L; Wisner, David H; Holmes, James F, 2016; S. Wang et al., 2017). Sexual dimorphism has also been noted in other species, and adrenals from female mice are significantly larger than their male counterparts (Bielohuby et al., 2007). These findings raise the question to what extent sex influences cellular turnover and stem cell biology. Using bromodeoxyuridine (BrdU) labelling, it was shown that steroidogenic cell proliferation was much higher in the female cortex, especially in the outer ZF (Chang et al., 2013; Grabek et al., 2019). The enhanced proliferation rate was also confirmed in lineage-tracing experiments, which demonstrated that in females the entire cortex is replaced every 3 months, whereas in males the renewal processes take up to 9 months (Dumontet et al., 2018; Grabek et al., 2019). To counterbalance this higher proliferation rate, the female cortex also showed a significantly higher amount of apoptosis (Grabek et al., 2019).

Sex not only affects the proliferation rate of steroidogenic cells, but also influences recruitment of progenitors. As described above, the definitive (adult) cortex is formed through the recruitment of *Gli1*$^{+}$ capsular cells. During puberty, however, capsular recruitment ceases almost entirely in male mice, and in adults only the occasional capsular cell being converted into a steroidogenic cell. By contrast, in female mice, capsular recruitment appears to be maintained throughout life. Sex-specific cell proliferation and recruitment does not depend on sex chromosomes (XX or XY) but is rather governed by sexual hormones (Grabek et al., 2019).

The observed higher cellular proliferation rate in female adrenals increases the risk of accumulating mutations. It is therefore likely that the sex-specific differences in adrenal cortex renewal are linked with the higher incidence of adreno-cortical cancer in women (Audenet et al., 2013a).

Harvesting developmental knowledge for therapeutic approaches

Adrenal insufficiency, such as Addison's disease, is a life-threatening condition that is caused by an absence or too low levels of hormone production. In most cases (80%) this condition is caused by adrenalitis that involves immune cells attacking the hormone producing cells of the adrenal (Hellesen et al., 2018; Maclaren, 2001). Treatment of adrenal insufficiency usually involves hormone replacement therapies. Unfortunately, lifesaving hormone treatments are notoriously difficult to dose and cannot completely mimic the pattern of hormone release from the adrenal gland that is subject to circadian rhythm and other complex physiological feedback mechanisms (Lightman et al., 2020). The hope for the future is therefore to encourage regeneration of adrenal tissue from adult stem cells or to develop cell replacement therapies with steroid producing cells.

To generate steroid hormone producing cells researchers have made use of the fact that NR5A1 acts as a master regulator of the steroidogenic cell fate and can activate the steroidogenic program in heterologous cell types; including embryonic stem cells, human skin cells, urine-derived cells, blood cells or mesenchymal cells from adipose tissue (Crawford et al., 1997; Ruiz-Babot et al., 2018; Tanaka et al., 2020). When transplanted under the kidney capsule or the adrenal gland of mice, the cells survived, continued to produce steroidogenic enzymes and extended the life span of adrenalectomized mice (Ruiz-Babot et al., 2018). However, since the hormone producing cells are heterologous, they are unlikely to respond to physiological stimuli and faithfully recapitulate adrenal responses.

As an alternative to forced NR5A1 expression in heterologous cells, adrenal stem/progenitor cells have been proposed as a source for transplantation. In principal, stem cells could be either generated by directed differentiation of induced pluripotent cells (IPS), or directly isolated from adult tissues. Some success for directed differentiation of pluripotent stem cells into steroidogenic lineages has been reported (Gondo et al., 2004; Jadhav & Jameson, 2011; Tanaka et al., 2007; Yazawa et al., 2011), but

in most cases protocols also involved forced NR5A1 expression (Crawford et al., 1997; Gondo et al., 2004, 2008; Sonoyama et al., 2012). A recent study by Li et al. suggests that the differentiation into Leydig-like or adrenal-like cells depends on the ECM, with collagen 1 favouring the gonadal fate (L. Li et al., 2019). They further speculate that high cortisol levels in the medium may contribute to a more adrenal fate. *In vitro* differentiation without forced expression of NR5A1 has also been attempted using mouse pluripotent cells that have been first differentiated to the IM stage. Induction of some NR5A1 expression and Sertoli-like cell phenotype was achieved by the additional treatment of cells with factors involved in the cAMP/PKA pathway. After transplantation into mouse testis, cells integrated but their functionality could not be tested (Seol et al., 2018).

The isolation of adult progenitor populations has also been attempted, but the lack of specific molecular markers and the absence of culture conditions that allow *in vitro* expansion of cells have hampered progress in this area (Bornstein et al., 2020). In mice, however, Nestin+ cells that have been isolated using a GFP reporter, were successfully expanded and produced steroid hormones *in vitro* (Steenblock, Rubin de Celis, et al., 2018). To what extent these cells will survive in long-term engraftments and whether they will respond to physiological stimuli *in vivo* remains to be proven in further experiments.

Whereas stem cell therapies are promising therapeutic approaches to adrenal insufficiencies, they may be less suitable for diseases that are caused by an excess of steroid hormones, such as genetic causes of Cushing's syndrome (Vaduva et al., 2020). Indeed, all of the abnormally producing cells, as well as their progenitors, would have to be first eliminated before 'repaired' stem cells could be introduced (Section, 2000; Sharma and Nieman, 2011). However, when Cushing syndrome is caused by adrenal tumours, surgical removal of adrenals may anyhow be the preferred treatment. In these cases, transplantation of adrenal stem cells may represent a promising approach to restore adrenal cortex function.

Conclusions and perspectives

Research over the last 10 years has provided us with a much more detailed view of the cellular and molecular processes driving adrenal development and tissue homeostasis. It has become clear that tissue renewal employs cell populations and molecular mechanisms that are also central to embryonic development and these findings may hold the key to the development of future therapies. Despite this progress, a number of areas need to be further studied. The precise molecular signals that specify the AGP within the mesoderm, as well as those that provoke separation of the AGP into AP and GP need to be identified. This will not only resolve a long-standing enigma in developmental biology, but may help us to finetune protocols for directed differentiation of pluripotent cells into true adrenal progenitors *in vitro.*

Secondly, it will be important to better characterize stem cell populations of the adult cortex. The identification of specific molecular markers that can be used for FACS or MACS sorting would allow the isolation of these cells from adult human tissue. In parallel, protocols for the culture and expansion of stem cells *in vitro* will have to be developed. This will permit us to study these cells in detail, modify them (mutation correction using CRISPR/Cas approaches) and in the future perhaps use them for cell replacement therapies. Congenital adrenal hyperplasia (CAH), a disease caused by mutations at the *CYP21A2* locus (Merke & Bornstein, 2005; P. C. White et al., 1984), would be particularly amenable to this approach, as gene correction in stem cells could provide a permanent cure for these patients.

Thirdly, we will need to investigate the reasons for the surprisingly distinct proliferation rate and stem cell behaviour in the two different sexes. While we know that androgens are responsible for the suppression of proliferation and capsular recruitment (Grabek et al., 2019), it is unclear whether this hormone acts directly on adrenal cells, or whether it may indirectly impact stem cell behaviour. A detailed study of mice carrying an adrenal specific knockout for the androgen receptor (AR) could

answer this question (Gannon et al., 2019). More importantly, it will be essential to understand the biological reasons for the differences found in the two sexes. By studying these questions, we may better understand the striking sex bias observed in adrenal diseases which in the long run will also lead to better treatment options. In addition to the importance for endocrinology and adrenal biology, such studies may also serve as a paradigm for other organ systems in which sex bias has been reported.

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1.1.b. Adult adrenal cortex.

During the maturation of the fetal adrenal cortex already the cells of DZ and TZ start to resemble their future lineages in the adult cortex, the zona glomerulosa (zG) and the zona fasciculata (zF) accordingly (Sucheston & Cannon, 1968). zG is the outer layer of the cortex consisting of ovoid cells, and it is followed by zF of large, organized cells and it makes for the largest portion of the cortex (**Figure.1**) (Nishimoto et al., 2010; Xing et al., 2015). In contrast, FZ cells of the human adrenal cortex will undergo apoptosis after birth, and the zone will slowly diminish in size and functionality (Ishimoto & Jaffe, 2011). In rodents, these cells will regress to the so-called x-zone, which will in turn disappear during the male puberty and after the female's first puberty (HOWARD & BENUA, 1950; TANAKA & MATSUZAWA, 1993). In humans, around the age of six to nine years old, the zona reticularis (zR) appears in between the zF and the medulla, in an event called adrenarche (Havelock et al., 2004). Each zone of the adrenal gland contributes differently to the steroidogenic producing function of the cortex, using as main ingredient the paracrine cholesterol (Mitani et al., 1999; Xing et al., 2015). zG produces mineralocorticoids, and the primary one is aldosterone, which influences the ion channels at target tissues by increasing the intake of sodium in exchange of potassium and hydrogen (JOHNSON et al., 1957; LARAGH & STOERK, 1957). zF releases glucocorticoids under the control of ACTH from the anterior pituitary (DEANE & BERGNER, 1947; HILLS & THORN, 1948; Mason et al., 1947), which are responsible for the energy management during stress, and modulate in turn the ACTH secretion from the HPA axis (Bellamy & Leonard, 1964; Ortega et al., 1976; SIE et al., 1964; Storrs, 1979). The main glucocorticoid in human is cortisol (INGLE et al., 1948), and in rodents is corticosterone due to the lack of Cyp17 gene (BANDY et al., 1956; Halberg et al., 1959; Halberg & Haus, 1960). zR produces androgen precursors, like DHEA and androstenedione (Conley et al., 2004; HYATT et al., 1983), where they will mature in nearby tissues into testosterone (**Figure.1**) (Kaufman et al., 1990; Pelletier, 2008; Rosenfield, 2005). This event can initiate the appearance of secondary sexual characteristics in primates, like development of pubic hair and altered sweat composition, followed by increased oiliness of the skin and hair (Rosenfield & Lucky, 1993).

After the adult zones are established, they are maintained by progenitor cells residing mainly bellow the capsule (A. C. Kim et al., 2009; King et al., 2009; M. A. Wood et al., 2013b). The cells of the adrenal cortex have a proliferation grading, with the proliferative cells being in the outer layers and cell death occurring near the medulla through migration (Morley et al., 1996; Salmon & Zwemer, 1941; Sasano et al., 1995; Stachowiak et al., 1990). As the fetal, the adult adrenal cortex is also highly vascularized with big arteries reaching its surface and branching to form thin capillaries throughout the cortex, in between the cells (LEVER, 1952). In principle, there are three major arteries reaching adrenal cortex: the superior suprarenal artery coming from the inferior phrenic artery, the middle suprarenal artery arises from the abdominal aorta, and the inferior suprarenal artery connecting with the renal artery (Wright & Burns, 2021). In first, this high vascularization serves for the hormonal homeostasis of the organism (Bornstein & Vaudry, 1998), but it also patterns the cells of each zone through the gradients

of steroids found through the cortex (ANSON & CAULDWELL, 1947; GAGNON, 1956; LEVER, 1952; Monkhouse & Khalique, 1986). Lastly, the adrenal cortex is highly innervated with the highest concentration of them at the medulla, but branches can be found alongside the blood vessels throughout the cortex (Engeland, 1998). Lately a population of cells related with neuronal lineage has been identified in the cortex, as a potential steroidogenic progenitor under stress conditions *in vitro* (Steenblock, de Celis, et al., 2018; Steenblock et al., 2017).

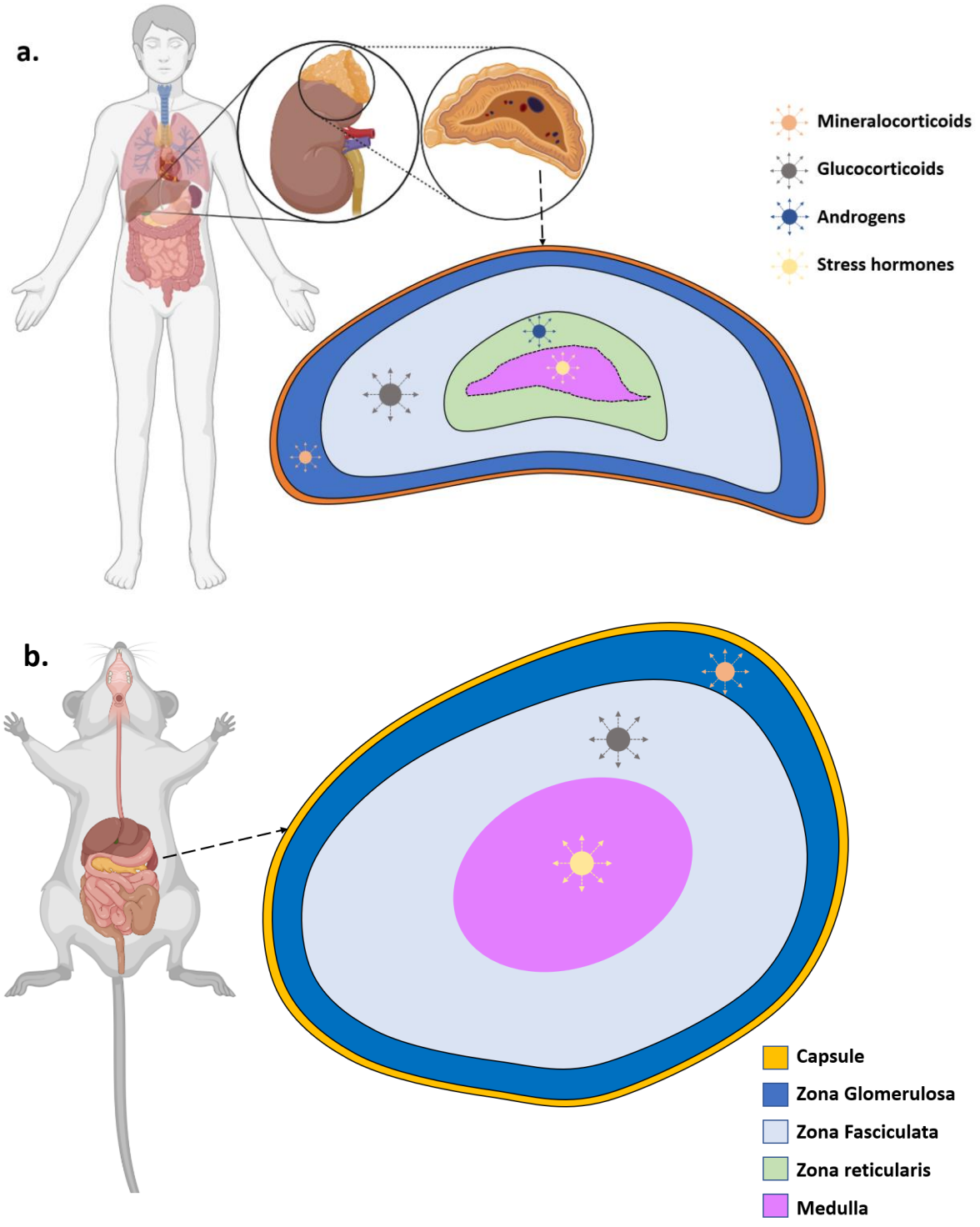


Figure.1. The adrenal cortex is located towards the back of the animal on top of the kidney on both human (a) and mouse (b). The adult adrenal cortex is composed with the capsule in the outer layer, as source of progenitor cells for the adrenal

cortex. Moving inward there is zG, zF and in humans zR, followed up by medulla. zG produces mineralocorticoids with the main one being aldosterone. zF makes up the biggest part of the adrenal cortex (~75% of its weight) and is a source of glucocorticoid production; cortisol and corticosterone as the primary one in human and mouse respectively. zR mainly produces sex hormones and predecessors of them. The medulla will produce the stress response hormones of Epinephrine (adrenaline), norepinephrine and dopamine.

1.1.c. Adrenal hypoplasia.

In pathological adrenal hypoplasia, the patients are characterized by insufficient steroidogenic production due to developmental issues or hypotrophism. There are two types of adrenal insufficiency. In primary adrenal hypoplasia, patients are incapable of perceiving the ACTCH signalling from the HPA axis, either due to ACTH related genetic disease or by adrenocortical developmental defects, which is classified as AHC (adrenal hypoplasia congenita). The latter is a more severe disease, linked with sexual and general homeostatic impairments (Arboleda et al., 2012; Habiby et al., 1996; Mandel et al., 2008). The genetic ACTH resistance, or familial isolated glucocorticoid deficiency syndromes, are caused by mutations on ATCH signalling cascade related genes. The most prominent mutated genes are its receptor (MC2R) and the accessory protein of the receptor (MRAP), which also have a part in fetal adrenal cortex homeostasis (Clark et al., 1993; Gineau et al., 2012; C. J. Kim et al., 2009; Novoselova et al., 2018; W. H. Yang et al., 2010). The most common AHC is the X-linked congenital adrenal hypoplasia, which affects predominantly males, and it is caused by Dax-1 mutations; other cause of AHC can be NR5A1 related mutations (Achermann et al., 1999, 2001; Burris et al., 1996; Habiby et al., 1996; Kawabe et al., 1999). It is important to mention another type of primary insufficiency, Addison's disease, a rare disease where the patient does not produce enough cortisol and aldosterone due to adrenal hypofunction (Hellesen et al., 2018; Kong & Jeffcoate, 1994; Maclaren, 2001). In secondary adrenal hypoplasia there is disruption in ACTH synthesis, maturation or/and secretion, resulting in a hypotrophic and hypofunctioning zF due to absence of signalling cues; this though can have consequences to other organs and not only the adrenal gland, because of the absence of paramount endocrine molecules (Jackson et al., 2003; Krude et al., 1998; Lefebvre et al., 2016; Novoselova et al., 2019).

1.1.a. Steroidogenesis.

Steroidogenesis is the biochemical procedure where cholesterol is imported into the steroidogenic cells and then subsequently to the mitochondria, from where its enzymatic conversion toward steroid hormones begins (Jefcoate et al., 1992; Miller & Bose, 2011; Payne & Hales, 2004). It's worth noting that cholesterol molecules mainly have to be imported into the cells as an active steroidogenic response, because steroidogenic cells store few amounts of these molecules, a procedure which is tightly controlled (Garren et al., 1965; Maxfield & Wüstner, 2002; Tontonoz et al., 1993). After entering the cell, cholesterol must be imported from the outer to the inner mitochondrial membrane, from where the enzymatic process of it and thus the production of steroid hormones will begin (**Figure.A1**) (Miller & Bose, 2011; Payne & Hales, 2004). The steroidogenic acute regulatory (StAR) protein is majorly induced by PKA and ERK pathway after appropriate signal in steroidogenic cells, and it is the one responsible for the importation of cholesterol (Arakane et al., 1997; Duarte et al., 2014; Poderoso et al., 2008; Pon et al., 1986; Pon & Orme-Johnson, 1986). The first enzymatic modification happens from CYP11A1, an enzyme of CYP or P450 family, by cleaving the cholesterol and thus creating pregnenolone, and marking the first rate-limiting step of steroidogenesis (Burstein & Gut, 1976; Shikita & Hall, 1973a, 1973b; Stone & Hechter, 1955). The expression of this enzyme in the

steroidogenic cells, like the rest of CYP family, requires the action of NR5A1 (K. Morohashi, 1999; K. Morohashi et al., 1992; Parker, 1999; Parker & Schimmer, 1995). In conjunction, PKA pathway and pituitary hormones actions through cAMP also induces the expression of these enzymes (John et al., 1984; C. C. Moore et al., 1990; Waterman, 1994), and other steroidogenic enzymes (LaVoie & King, 2009), but further factors can optimize their expression (Payne & Hales, 2004). *Cyp11a1* is expressed early at the development of AGP at E10.5 of mouse (Ikeda et al., 1994), followed by a stronger expression in AP than in gonads in mouse (Ikeda et al., 1994), human (Voutilainen & Miller, 1986) and rat (Rogler & Pintar, 1993). From there it will be gradually expressed in FZ, TZ and then DZ (Mesiano et al., 1993), and finally to the postnatal adrenal cortex, where its expression is paramount for the organism (M.-C. Hu et al., 2002)

CYP21 serves as the catalytic step for the production of steroid hormones with adrenal origin; this step takes place in the ER of the cells (Payne & Hales, 2004; Wijesuriya et al., 1999). As CYP21 marks the start of adrenal specific steroidogenesis (Wijesuriya et al., 1999), it is expressed specifically in the adrenal tissues (Parker et al., 1985). Interestingly, there are two genes encoding this enzyme, from which *Cyp21a* transcribes the active enzyme in mouse and *CYP21B* in human (Parker et al., 1986). The expression pattern in the fetal adrenal gland follows the same as the one mentioned for *Cyp11a1* (Narasaka et al., 2001) and postnatal expression in human is mainly observed in ZF and ZG (Coulter & Jaffe, 1998). Following the enzymatic step of CYP21, the adrenocortical steroidogenesis finalizes in the inner mitochondrial membrane by CYP11B enzymes; CYP11B1 and CYP11B2 (Mornet et al., 1989). CYP11B1 is responsible for catalysing the glucocorticoids to corticosterone/cortisol (human) and CYP11B2 the mineralocorticoids to aldosterone (Mornet et al., 1989). Both enzymes have specific expression in the adrenal cortex where the follow the steroid production pattern of the adrenocortical zones, with CYP11B1 found mainly in ZF and ZR, while CYP11B2 having a restricted expression in ZG (Åkerström et al., 2016; Curnow et al., 1991; Ogishima et al., 1991). During the human fetal adrenal development, both CYP11B1 and CYP11B2 are expressed high in FZ and lesser in TZ, but absent in DZ (Coulter & Jaffe, 1998).

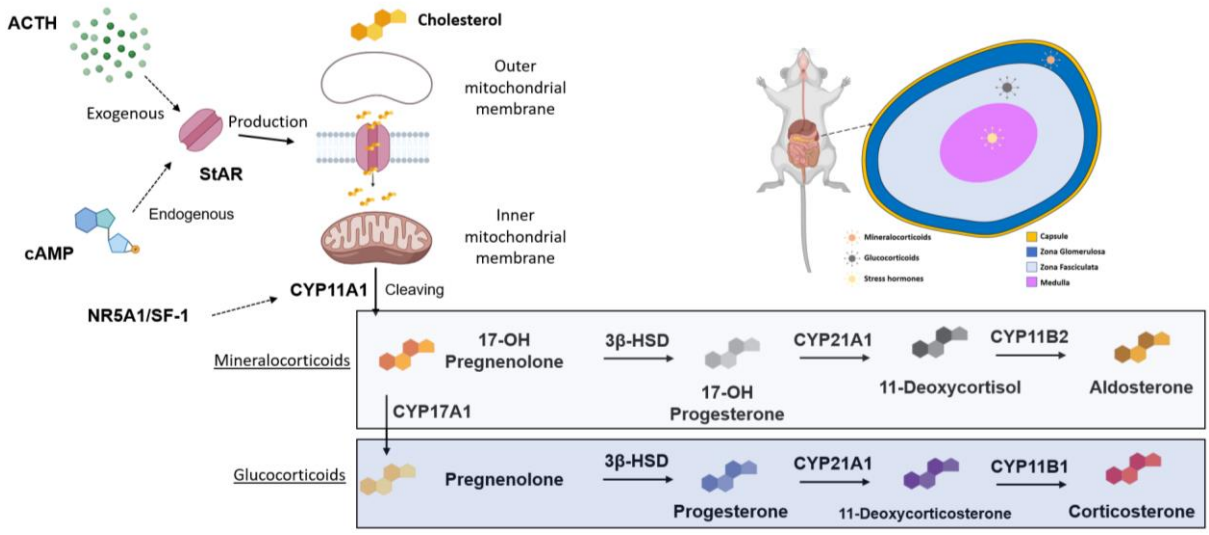


Figure.A1. Graphical depiction of steroidogenesis in mouse adrenocortical cells. The steroidogenesis begins with the importation of Cholesterol from the outer to the inner mitochondrial membrane through StAR; StAR is also activated in response to steroidogenic signal. Thereafter Cholesterol will be cleaved for first time by CYP11A1, and it will be continuously

processed by enzymes, with final product of either Aldosterone or Corticosterone/Cortisol (human), according to the cell identity. The expression of the steroidogenic enzymes falls also under the control of NR5A1 and ACTH/cAMP induction.

1.1.d. Adrenal hyperplasia.

Hyperplasia is related with the excessive growth of a tissue, and in this occasion are divided into primary and secondary. Primary adrenal hyperplasia is believed to be ACTH independent, but there are results who show that ACTH stimulus may be involved (Louiset et al., 2013; Merke & Bornstein, 2005). Secondary adrenal hyperplasia is a result of ACTH overstimulation, which then can be further divided based on the pathology. Congenital adrenal hyperplasia (CAH) is caused by a group of autosomal disorders, which lead to overaccumulation of steroid precursors and the subsequent enlargement of the adrenal. The most common cause of CAH is a null mutation of 21-hydroxylase (CYP21A2) enzyme, in which the fetal adrenal cortex cannot balance the production of cortisol resulting to defective communication with the pituitary and further genetic malformations (Carvalho et al., 2020; Morel & Miller, 1991; Speiser et al., 2010; P. C. White et al., 1984). Patients with CAH can be distinguished in their birth if they have the classical form or they can have an unnoticeable hyperandrogenism until adulthood in the non-classical form (Nimkarn et al., 2011; Nimkarn & New, 2007). CAH patients can have mutations in other steroidogenic related genes, like STAR, CYP11A1, HSD3B2, CYP17A1, CYP11B1, and POR, where they have different phenotypes but same cause (Hauffa & Hiort, 2011; Krone & Arlt, 2009; Lekarev et al., 2012).

The hyperplastic adrenal tissue due to tumorigenesis can be either non-functional, or functional by overproducing enzymes. Most commonly, a steroidogenic producing hyperplastic manifestation, or unilateral adrenal adenoma or carcinoma leads to Cushing's syndrome (Ghayee et al., 2011; HAYASHI et al., 1998; Lacroix et al., 2015). Cushing's syndrome is caused by chronic exposure to accumulative amounts of adrenal produced glucocorticoids and can both ACTH dependant (Cushing's disease) or independent, with the latter being more common to females (Lacroix et al., 2015). In more rare cases there is overproduction of mineralocorticoids and androgens (Ghayee et al., 2011); in both cases, these hormonal overproducing manifestations are coupled with abnormal activity of the PKA pathway (Espiard et al., 2014; Lerario et al., 2014). On the other hand, unilateral adrenocortical tumours (ACTs) are a common event on aging individuals. Most commonly these adrenocortical adenomas are non-functional, but there are cases where they produce cortisol (Cushing syndrome), aldosterone (Conn syndrome), or androgens (Arnaldi & Boscaro, 2012; Melvin M Grumbach et. al, 2003).

1.1.e. Adrenal diseases.

Adrenal diseases tent to be sexual dimorphic, including some of the mentioned above like AHC, Cushing's and Addison's (Achermann et al., 2000; Audenet et al., 2013b; Lyraki & Schedl, 2021a), although there is still more to understand on the homeostasis of those diseases (Lyraki & Schedl, 2021b). Nevertheless, stem/progenitor cell factors critical for proper adrenal development and homeostasis like Wnt/beta-catenin and insulin signalling are the most common dysregulated pathways in adrenal hypoplasia, hyperplasia, and tumours (Xing et al., 2015). This fact also promotes the link between developmental processes and tumorigenesis, since the same pathways can either lead to hypoplasia or hyperplasia, in their absence or overactivity accordingly (Ferraz-de-Souza et al., 2011; Xing et al., 2015). Thus, the understanding of the adrenocortical development can lead us to further understanding the source of its genetic diseases, but also the cancerogenesis and possible

cures; this logic can be translated to other tissues and organs (Oikonomakos, Weerasinghe Arachchige, et al., 2021; Penny et al., 2017; Xing et al., 2015). Stem cell research will be beneficial for these patients via tissue and tissue regeneration or by finding more suited drug therapies through drug screening in *in vitro* models (Bornstein et al., 2020; Lyraki & Schedl, 2021a; Oikonomakos, Chamara, et al., 2021).

1.2. Tools in regenerative medicine.

1.2.a. Pluripotent Stem Cells.

Stem cells have the ability of continuous generation of two types of daughter cells, one that has the same potency as them and another able to commit in further differentiation processes (Martello & Smith, 2014). Pluripotent stem cells (PSCs) are marked by their ability to self-renew and potentially give rise to any cell in the body, besides tissues of extra-embryonic origin. Embryonic stem cells (ESCs) are pluripotent stem cells, they originate from the inner cell mass (ICM), where in mouse they can be harvested between E3.5-4.5 (Martello & Smith, 2014). Pluripotency can be divided into naïve or prime, depending on the characteristic of the cells. Naïve PSCs have high self-renewal ability, they can contribute to pre-implantation stage blastocysts by chimerism, and they form round shaped colonies when cultured; mouse ESCs are in naïve state. Primed PSCs are more easily differentiated and when cultured they have more flattened colonies, resembling post-implantation state; human PSCs and mouse epiblast stem cells (EpiSCs) are in this state (Nichols & Smith, 2009) (**Figure.2**).

The knowledge of ESCs started from an inbred mice line in which they observed and later transplanted teratocarcinomas, a type of tumour which has a blend of different tissues, something that in a notable but abstract way resembles the normal development (Stevens & Little, 1954). In another study, embryonic carcinoma cells (ECCs) were transplanted in mice brains, and they were able to generate different types of teratocarcinomas, which shows the dynamism of these cells (KLEINSMITH & PIERCE, 1964). In a continuity, further transplantation of these tumours showcased that the differentiated cells of the teratomas do not have the ability of self-renewing and propagating, but this is a characteristic of the ECCs (G. R. Martin, 1980). To further validate the potency of cells, mice embryos were transplanted outside of the uterus and the pluripotent cells gave rise teratocarcinomas (Diwan & Stevens, 1976; Solter et al., 1970; Stevens, 1968). Thereafter, ECCs were able to create chimeric mice after their implantation in the developing mouse embryo, thus displaying the ability of a subset of ECCs to respond and integrate to the embryonic environment (Papaioannou et al., 1975). At the same time, culturing of ECCs was established by using the protocol of primary cell culture where cells are seeded on inactivated feeder cell layer (Kahan & Ephrussi, 1970). Interestingly, when ECCs were aggregated, they formed 3D structures partially resembling features of early embryos; these structures were termed embryoid bodies (G. R. Martin & Evans, 1975).

With the gathered data and knowledge from ECCs, people manage to apply them in order to culture pluripotent cells directly from embryos (Evans & Kaufman, 1981; G. R. Martin, 1981). These pluripotent cells in the end were identified as the ESCs, and one of the main features is their ability to highly contribute to chimeras (Bradley et al., 1984). ESCs can maintain their pluripotency through the cell passages with the addition of leukaemia inhibitory factor (LIF) (A. G. Smith & Hooper, 1987; R. L. Williams et al., 1988), and with the addition of Mek/Erk inhibitor (PD0325901) and GSK3b inhibitor (CH99021) (Ying et al., 2008). It has been reported that cultured XX ESCs tend to lose one of their X chromosomes in prolonged cultures (Rastan & Robertson, 1985). These methods allow reliable clonal expansion and new derivation of undifferentiated ESCs from blastocysts, even without the help of

feeder cells (R. L. Williams et al., 1988; Ying et al., 2008). On the other hand, EpiSCs can be also cultured and differentiated further, but with the addition of FGF and Activin instead of LIF and the 2 inhibitors (2i), and they cannot integrate to the blastocyst (Brons et al., 2007; Kojima et al., 2014; Tesar et al., 2007).

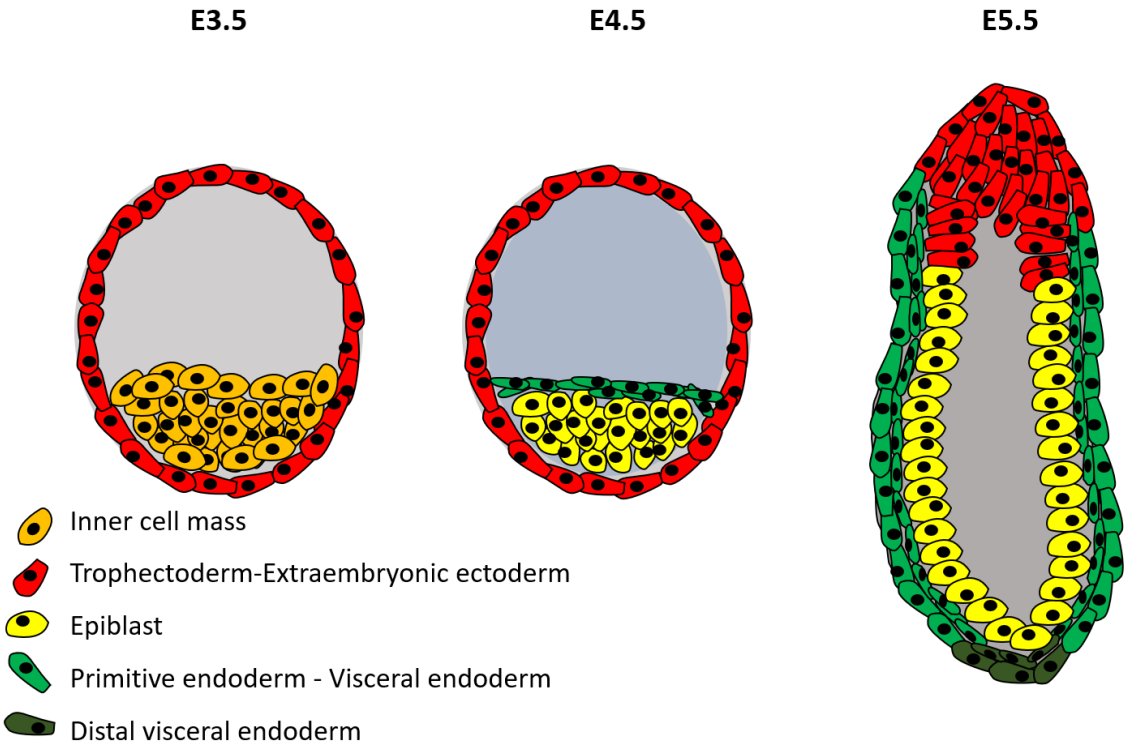


Figure.2. Abstract depiction of early mouse embryonic development. At E3.5 the source of naïve PSCs (ICM) is present, surrounded by trophectoderm. At E4.5 ICM is defined to Epiblast, source of naïve PSCs, and primitive endoderm. After the implantation of the mouse embryo, at E5.5, there is the extraembryonic ectoderm and visceral endoderm, with the appearance of distal visceral endoderm; the epiblast cells are now a source of primed PSCs.

The first core pluripotency factor identified and characterised was the POU-domain transcription factor *Oct4 (Pou5f1)/Oct3* (Okamoto et al., 1990; Schöler et al., 1990), where its deletion causes the cells to differentiate and lose the pluripotency in the ICM (Nichols et al., 1998). The paramount factor for their self-renewal has been identified as the SRY-box transcription factor *Sox2* (Masui et al., 2007), and together with *NANOG* they create the triad which governs the pluripotency of ESCs through cross positive feedback with each other. Although NANOG is dispensable, overexpression of any of these genes leads to differentiation (Young, 2011). When ESCs differentiate physiologically, they start losing the expression of these three genes and they enter to a reversable transitional stage, and gradually they start upregulating lineage specific factors (Martello & Smith, 2014); they are also very sensitive to external signalling (Hayashi et al., 2008). These features characterise the plasticity of PSCs and their potential to perform guided differentiation under the proper signalling cues *in vitro* (Hayashi et al., 2008; K. M. Loh et al., 2014, 2016).

1.2.b. Induced Pluripotent Stem Cells.

Mice and mESCs are a powerful model for deciphering developmental and disease processes, but hESCs are in many aspects different from mESCs (Rao, 2004). Although, generation and manipulation of human ESCs is ethically debatable, which limits the potential and their application in regenerative

medicine and halts our further understanding of human development. This issue was answered by the successful generation of induced pluripotent stem cells (IPSCs) from mouse fibroblasts (K. Takahashi & Yamanaka, 2006) and later from adult human fibroblasts (K. Takahashi et al., 2007). This method allows the transformation of committed adult cells to pluripotent cells with the ability to give rise to all embryonic tissues, by direct reprogramming them with ectopic expression of OCT4, SOX2, KLF4 and cMYC (OSKM) (Maherali et al., 2008; Okita et al., 2007; K. Takahashi et al., 2007; K. Takahashi & Yamanaka, 2006; Wernig et al., 2007). Although the reprogramming period takes longer for human derived fibroblasts (Maherali et al., 2008; Okita et al., 2008). The assessment of IPS pluripotency is done, like it was already mentioned for PSCs, with the ability of them to either create teratomas upon transplantation or contribute to chimerism (Okita et al., 2007; Wernig et al., 2007). MIPSCs have been shown to be extremely potent, since they are able to generate the whole mouse during embryogenesis through tetraploid complementation (Boland et al., 2009; Kang et al., 2009; X. Zhao et al., 2009). Interestingly, IPSCs keep their epigenomic integrity *in vitro* for several passages or keep it forever when they are derived from mice or humans respectively, and they are more prone to commit to their original tissue lineage during in *vitro* differentiation (K. Kim et al., 2010, 2011). So far, IPS from different tissues of origin has been generated in lab, like: stomach cells, liver cells, T cells, B cells, hematopoietic stem cells, keratinocytes, melanocytes, mesenchymal stem cells, epithelial cells, cord blood, amnion cells, neural stem and progenitor cells (Aoi et al., 2008; Cai et al., 2010; Eminli et al., 2008; Hanna et al., 2008; Y.-H. Loh et al., 2009; Maherali et al., 2008; Okita et al., 2013; Seki et al., 2010; Sumer et al., 2011; Utikal et al., 2009; T. Zhou et al., 2011) (**Figure.3**).

The IPSCs have been used very shortly after their creation for drug screening and optimization (Lee et al., 2009), and in order to create tissue samples for disease modelling (Siller et al., 2013) (**Figure.4**). Researchers are also translating protocols between mouse and human models, this creates a greater dynamism and flexibility to the system (Chow et al., 2020; Lindström et al., 2018; Taguchi & Nishinakamura, 2017; Takasato et al., 2014) This information showcases the immense potential for IPSCs in disease modelling, regenerative and personal medicine (Rufaihah et al., 2021). Advances in *in vitro* culture allows to culture cells in xeno free (XF) and in feeder free (FF) conditions, through defined and sophisticated cell medium and ECMs for the cells to grow; thus, setting the cells for clinical use (Braam et al., 2008; K. G. Chen et al., 2014; Nakagawa et al., 2014; Zujur et al., 2020). Indeed, since then researchers have moved to apply their knowledge with the combination of this tool in clinical trials for potential stem cell therapies (Trounson & DeWitt, 2016). A concern for stem cell therapies has always been the patient immune reaction to donor cells, thus researchers use patient derived IPSCs (Umekage et al., 2019). The first reports of successful use of hIPSCs in therapeutic treatment has been already validated (Mandai et al., 2017), with more potential hIPSCs treatments on the way, like neuronal (Kikuchi et al., 2017; Morizane et al., 2017) and heart diseases (Funakoshi et al., 2021; K.-L. Wang et al., 2021) and even alternative methods to battle cancer (Barber et al., 2006; Nishimura et al., 2013). Although if the differentiation protocols are not defined for the cell line, the produced cells will not be of pure population which can lead to even tumorigenesis if the cells are still in pluripotent state Researchers are optimizing these conditions and they aim to ensure the safety of the hIPSCs before applying them to human therapies (Ghosh et al., 2011; Jo et al., 2020; Kuang et al., 2017; Tani, 2015).

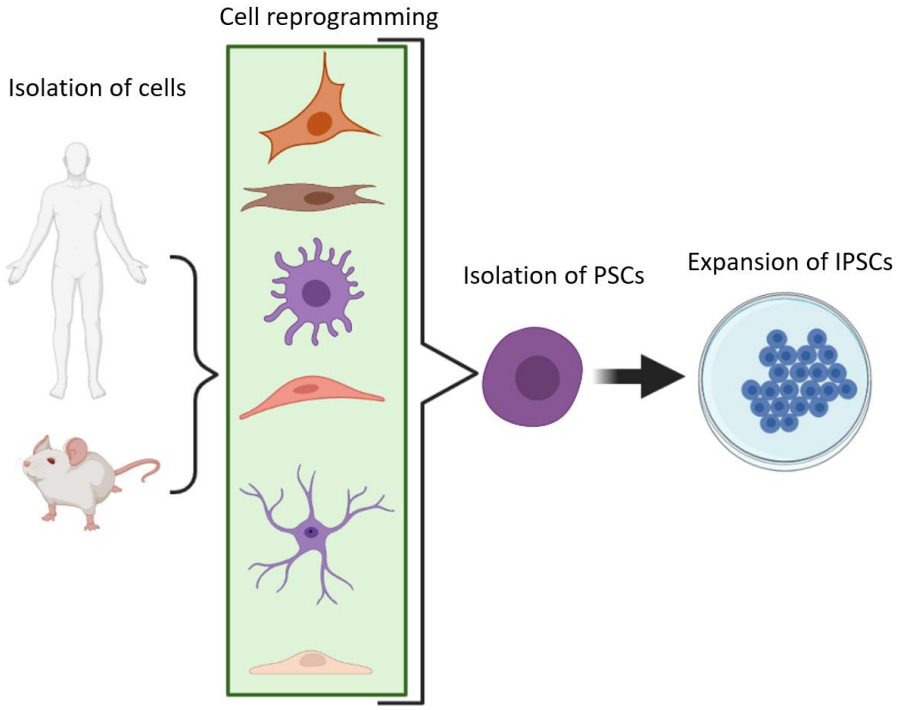


Figure.3. Defined adult cells are isolated by the host and cultured *in vitro*. The cells then can be reprogrammed into a state of greater potency by any of the scientific protocols, like inducing their pluripotency with the introduction of the Yamanaka factors (OSKM). The PSC colonies are then isolated based on their phenotype, then analysed for their state and they can be further expanded in culture and through different cell passages.

1.2.c. Gastruloids.

The advances in stem cell therapies are based greatly on the progress researchers have made over the years in understanding embryonic development and tissue physiology, thus recapitulating it *in vitro* for both mouse and human (Murry & Keller, 2008; Rufaihah et al., 2021). This bridging was made possible by analysing microenvironments which mimic the normal development (Simunovic & Brivanlou, 2017; Vianello & Lutolf, 2019). ESCs can be aggregated to form embryonic bodies and assessing the embryonic development under controlled and monitored environment (Brickman & Serup, 2017; Vianello & Lutolf, 2019), defining their environment greatly aided in further development (Shao et al., 2017). If left alone these 3D structures anarchically create differentiated tissues, but they can be controlled with the appropriate signalling cues (Beccari et al., 2018; Moris et al., 2020; Veenvliet et al., 2020). In the very basis, PSCs can replicate events in early gastrulation under correct stimuli and fate the cells into define cell lineages (Camacho-Aguilar & Warmflash, 2020; Minn et al., 2020). These methods of *in vitro* modelling can provide sophisticated templates for the early developmental stages, necessary to drain knowledge out of them, especially for human embryogenesis (Fan et al., 2021; Hadjantonakis et al., 2020). The 3D structures recapitulate the gastrulation events *in vitro*, it has been termed gastruloids, and for their creation they rely mostly in endogenous signalling after their initial creation steps (Moris et al., 2020). These structures express markers of the derivatives of the three germ layers and a patterning as in a vertebrate embryo at that stage, and symmetry plan (Beccari et al., 2018; Moris et al., 2020). Thus, they have the potential to create more functional tissues out of them or model the development of specific tissues (Moris et al., 2020), something which has been illustrated recently for early human cardiogenesis (Hofbauer et al., 2021).

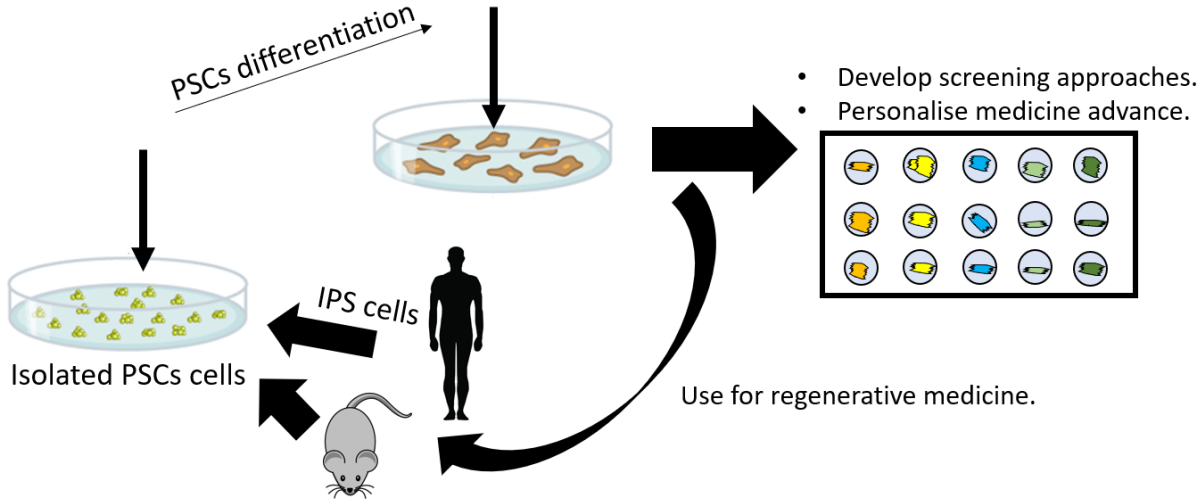


Figure.4. PSCs can be cultured under defined conditions either by isolation of ESCs or by making IPSCs. The PSCs then can undergo targeted differentiation *in vitro* to the tissue of interest. From there, depending on the needs, the forming cells will either get more defined for use in regenerative medicine (tissue replacement, repair etc.) or they will be used as screening tools for diseases and drug development in the form of organoids or in vitro functional tissues.

1.3. Embryonic development.

1.3.a. Gastrulation and Primitive streak formation.

Before gastrulation, around E5.5 of mouse embryo, there are three epithelial population: the pluripotent cells of the epiblast (EpiSCs), the extraembryonic ectoderm (ExE) and the visceral endoderm (VE). The rodent embryos at this point are orientated in a symmetrical cup-shape, which differs from the disc-shaped of human and avian. In the embryonic development of chordate there are similarities and differences in geometry and patterning, but the basic procedures remain conserved (Garcia-Martinez & Schoenwolf, 1993; Keegan et al., 2004; Kinder et al., 2001; Martyn et al., 2019). A population of these endothelial cells will gradually transit to mesenchymal cells, in a process termed EMT (endothelial to mesenchymal transition) (Rivera-Pérez & Hadjantonakis, 2015). The gastrulation, which in mice lasts from embryonic day (E) 6.25 to E9.5, will give rise to the three germ layers: ectoderm, mesoderm, endoderm (P. P. L. Tam & Behringer, 1997a). Gastrulation initiates with the formation of primitive streak (PS) and the neuroectoderm (Bautzmann, 1927), and it is strictly dependant on the proper orientation and division of the epithelial populations (Rivera-Pérez & Hadjantonakis, 2015). The PS will act as a signalling beacon and will mark where the EMT occurs through the gastrulation, which subsequently leads to the formation of mesoderm and endoderm (**Figure.5**) (Stern & Laboratory, 2004). A structure termed as the organiser, which later gives rise to notochord and ventral neural tube, it is a focal point for axial orientation of the embryo. From gastrulation until the somitogenesis the embryo continuously elongates to the anteroposterior axis (A-P). During the elongation there is a continuous signal secretion, proliferation, and migration of cells, which forms proper niches for fating the differentiation of the germ layers and their progenitor cells throughout the embryo (C. Anderson & Stern, 2016; de Robertis, 2006; P. P. L. Tam et al., 1993; M. Williams et al., 2012; Wilson et al., 2009).

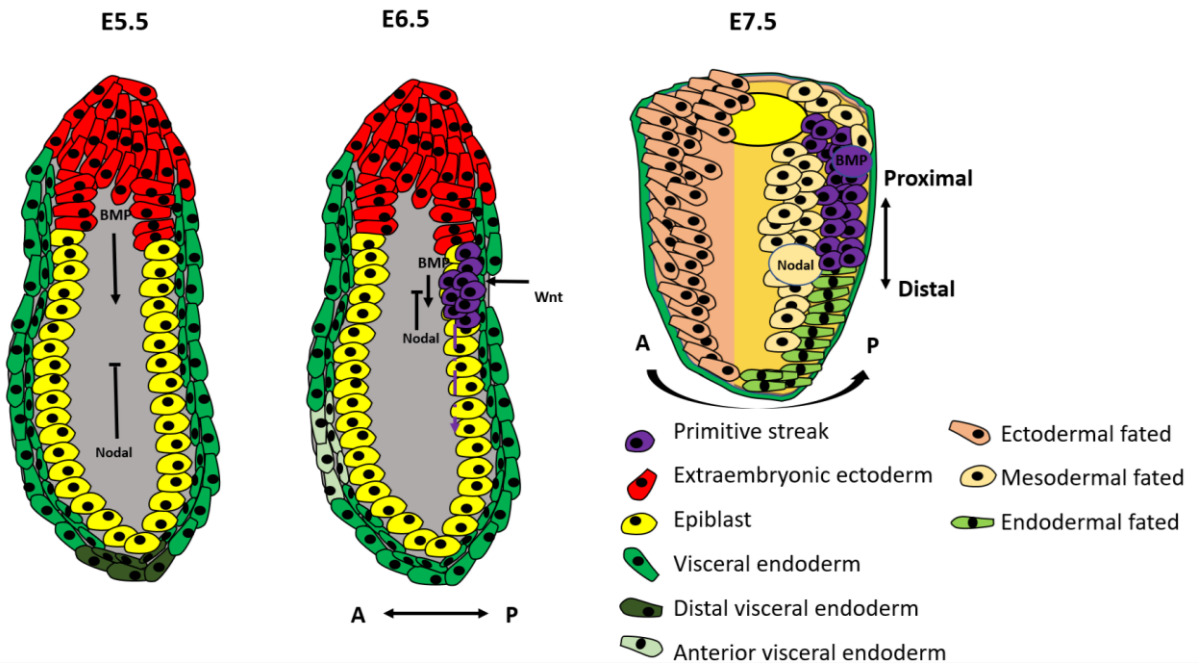


Figure.5. Synopsis of fated map during early gastrulation of mouse embryo. Due to signalling gradients before gastrulation (E5.5) some epiblastic cells are fated to neuroectodermal and PS fate. The gastrulation begins at E6.5 with the appearance of PS cells which begin committing EMT, which cells will start expanding on A-P axis; at the same progenitors of neuroectoderm appear. Signalling from BMP is coming from posterior PS, and the expanding cells to the anterior axis expresses its antagonist, Nodal. Signals like Wnt and FGF pathway are also emitted by the PS and fate the exiting and surrounding cells. At E7.5, the neuroectoderm progenitors are forming their layer over the position of the epiblast, and the node has been established by the initial population of PS cells which acts as signalling hub and as axis point. Cells of the epiblast continue to undergo EMT when entering the PS and in their exit, they have become fated for their cell lineage. Cells in their exit from the PS will continue expanding to the A-P axis, forming a layer of cells over the epiblast and neuroectoderm. VE surrounds the forming embryo, and the extraembryonic tissues are on top of the epiblast.

Fate mapping of mouse and avian embryos showed that mesodermal progenitor cells are quickly fated, and their anteroposterior distribution in the PS reflects their future medio-lateral fate. Cells within the node generate the notochord, and then progressively from posterior to anterior axis of PS, extraembryonic, lateral plate, intermediate and paraxial mesoderm will be formed, followed by definitive endoderm on the anterior part. (Kinder et al., 2017; Lawson et al., 1991; Psychoyos & Stern, 1996; Schoenwolf et al., 1992; J. L. Smith et al., 1994; P. P. L. Tam & Beddington, 1987; Wilson & Beddington, 1996). Cells will begin to exit PS and differentiate timewise from the more posterior population, leading to the more anterior ones(Kinder et al., 1999; P. P. L. Tam & Behringer, 1997b). Some of these mesodermal progenitor populations of cells can be clearly pinpointed from E8.5 of mouse, showing the spatial importance for the developing niche in the embryo (Cambray & Wilson, 2002; Kinder et al., 2017; Wymeersch et al., 2016). Interestingly, clonal analysis experiments have demonstrated the existence of a population with dual-fated progenitor cells for neuroectoderm and paraxial/somatic mesoderm. These cells, termed neuromesodermal progenitors (NMPs), suggest a dynamic activity on the posterior end of the embryo, where there is an equal contribution to neural and mesodermal tissues (Tzouanacou et al., 2009). Indeed, it seems that there is a balance between the total commitment to one or the other germ layer, which is represented with the expression of *Tbx6* (mesodermal) and *Sox2* (neurectoderm) (Takemoto et al., 2011a). The specification of these progenitor cells and the initiation of neurogenesis signal the final stages of gastrulation, at around

E9.5 of mouse embryo, where ingression of new cells at the PS terminates (Cambray & Wilson, 2007; P. P. L. Tam & Behringer, 1997a; Wilson & Beddington, 1996).

The formation of PS is also depending on the expression of key genes, which also have a spatial expression profile, characterizing regions of the embryo. *Fgf5* is an early marker of gastrulation during epiblast state, and when it is depleted, mice embryos seem to lack gastrulation (Hebert et al., 1991). At this point, Sox2 is downregulated in PS and *Snail1* begins to express in the PS cells, and it promotes the EMT (Bazzi et al., 2017; Carver et al., 2001; Ramkumar et al., 2016). *Snail1* will continue to promote EMT until cells exit PS, mainly marking the mesodermal progenitor population (Bazzi et al., 2017; Carver et al., 2001). When the embryo fully forms a PS two distinct, and moderately overlapping, regions can be observed based on their gene expression of either *Foxa2* or *Brachyury* (T). The anterior PS will form the definitive endoderm, and it's marked with the expression of Foxa2. Its expression is also pivotal for the patterning of PS epiblast and neural tube (Ang & Rossant, 1994; Burtscher & Lickert, 2009). T on the other hand will mark the posterior region of PS. Its continuous expression is paramount for the correct formation of the later mesodermal tissues, and for the maintenance of Tbx6 expression which is also important for the specification of paraxial mesoderm (Chapman et al., 1996; Wilson et al., 1995). The region that co-expresses T and *Foxa2* will contribute to lateral plate and axial mesoderm, in early and late gastrulation respectively (Bardot et al., 2017; Burtscher & Lickert, 2009). T and *Tbx6* are markers of the mesodermal fated cells of PS and they have direct or indirect influence on their further morphogenesis and lineage commitment (Chapman & Papaioannou, 1998; Yamaguchi et al., 1999). Furthermore, the expression of specific receptors and downstream targets of signalling pathways are linked with proper development during the PS and on (B. G. Ciruna et al., 1997; Mishina et al., 1995; Yoshikawa et al., 1997).

During the whole process of embryogenesis, the cells are exposed to an array of signalling combinations depending on their spatial and chronical localisation. This results to three-dimensional map of the embryo with antagonistic signals (Peng et al., 2019). Starting from pregastrulation embryo, TGFβ, BMP, FGF and Wnt signalling pathways are necessary to drive cells to PS fate and omit EMT (Conlon et al., 1994; D. James et al., 2005; P. Liu et al., 1999; Mukhopadhyay et al., 2001; Vallier et al., 2005; Yamaguchi et al., 1994). Otherwise with either loss or inhibition of them, the epiblast cells quickly lose their pluripotency and are driven to neuronal fate by default; a characteristic well preserved evolutionary from amphibians to mammals (Bachiller et al., 2000; Glinka et al., 1998; Hemmati-Brivanlou & Meltont, 1997; Henry et al., 1996; Levine & Brivanlou, 2007; Smukler et al., 2006; Tropepe et al., 2001). Retinoic acid (RA) is also a potent inductor towards neuronal lineage, at this stage (Rhinn & Dollé, 2012). From the beginning of gastrulation, around E6.5 of mouse, the area of the forming PS is a hub of strong FGF, BMP and Wnt signalling, which are essential for the patterning of the whole embryo.

These signals will then go on and form an expression pattern map which will then form and fate the future endodermal and mesodermal tissues (Brennan et al., 2001; B. Ciruna & Rossant, 2001; Conlon et al., 1994; Huelsken et al., 2000; Kinder et al., 2017; P. Liu et al., 1999; Mishina et al., 1995). At the point of mid-gastrulation, around E7.5 of mouse, the PS will be polarized, and antagonistic signals will pattern on the A-P axis (Klaus et al., 2007; Robb & Tam, 2004), with the anterior PS strongly expressing the BMP antagonist, Nodal (Vincent et al., 2003). The anterior PS will remain a pole of Nodal expression through the elongation of PS, where at the end of it will continue to be expressed from the embryonic node (Conlon et al., 1994; Norris & Robertson, 1999; Vincent et al., 2003). On contrary, the

posterior PS will have high levels of BMP expression, and all the cells in A-P axis will be fated based on the balance of these two signals, with the future tissues being shaped in PS as mentioned above (**Figure.5**) (P. P. Tam & Beddington, 1992; Vincent et al., 2003; Wilson & Beddington, 1996; Winnier et al., 1995). Same polarizing A-P pattern with inductors and inhibitors this time, it is exhibited with Wnt signalling, and the combining time of exposition on these signals before exiting the PS will also attribute to the specification of the future tissues (Conlon et al., 1994; Kemp et al., 2005; Klaus et al., 2007; Saykali et al., 2018). FGF signalling has a governing role on fating cells of the anterior mesoderm by down-regulating E-cadherin and promoting their migration out of the PS and start the somitogenesis (B. Ciruna & Rossant, 2001; García-García & Anderson, 2003; Sun et al., 1999; Yamaguchi et al., 1994). Furthermore, there are indications that at this point hedgehog signalling is acting in collaboration with FGF signalling for the patterning of future mesodermal tissues (Guzzetta et al., 2020), but also, it has a pivotal role in forming a correct L-R patterning (X. M. Zhang et al., 2001). As the cells keep on differentiating and exiting the PS stage, they in turn will start producing new signals which further influence the environment of the embryo and form boundaries between the different stages of cells (Peng et al., 2019). Lastly, RA signalling is also contributing to the L-R symmetry during later gastrulation, antagonizing the FGF signalling and contributing to the proper somite formation (del Corral et al., 2003; Sirbu & Duester, 2006; Vermot & Pourquié, 2005), while also having a possible patterning effect on the PS derived tissues in a A-P matter (Hochgreb et al., 2003).

1.3.b. Mesoderm development.

The germ layer of mesoderm is the most recent which appears evolutionary, and it is highly represented in vertebrates (Technau & Scholz, 2003). This third layer has majorly specialised derived cells and it is associated with more elaborate functions such as reproduction, nutrient exchange, circulatory systems and being a supportive tissue for internal organs. As it has been described, mesoderm is subdivided into paraxial, intermediate, and lateral plate (R. G. James & Schultheiss, 2003), depending on signal ques mainly from BMP balance (**Figure.6**) (Pourquié et al., 1996).

1.3.b.i. *Paraxial Mesoderm.*

Paraxial is the more medium and closest to the notochord compartment of the mesoderm. Secreted Noggin from the notochord antagonises the lateral BMP signalling and promotes the fating of cells towards paraxial mesoderm through the embryonic development (**Figure.6**) (Pourquié et al., 1996; Tonegawa & Takahashi, 1998). The development of paraxial mesoderm can be traced in three stages: presomitic mesoderm specification, somitogenesis, and somite specification (Christ & Scaal, 2008).

The presomitic mesoderm, which is the earliest form of paraxial mesoderm, it is localised by the notochord as a group of mesenchymal cells (P. P. L. Tam & Beddington, 1987) and it derives from the PS or NMPs of the tail bud (Takada et al., 1994). The anterior part of the presomitic mesoderm will give rise to the somites. The periodicity of somitomere formation is produced by the segmentation clock that operates in the presomitic mesoderm, while simultaneously creating the future somatic boundaries, through dynamic signalling effects (Dubrulle et al., 2001). This process is heavily spatiotemporally regulated (Cooke & Zeeman, 1976), and the cells will undergo mesenchymal to epithelial transition (MET) to form somites (Burgess et al., 1996). Mature somites contain two major populations: the sclerotome and dermomyotome. To form the sclerotome, the ventromediuml somite cells will undergo EMT (Christ et al., 1978). The sclerotome will in turn give rise to the vertebrae and associated ribs, tendons, and tissues like the vascular cells of the dorsal aorta, intervertebral blood vessels, and meninge (Christ & Scaal, 2008; Nguyen et al., 2014). In turn the dermomyotome gives rise

to the myotome and the dermatome. The myotome produces the back, rib cage, ventral body wall, and limbs, while the dermatome gives rise to the dermis and, to some studies in chick embryos, contributes to muscle development (Ben-Yair & Kalcheim, 2005).

The presomitc mesoderm can be identified easily by the expression of *Tbx6*, after paraxial cells exiting the PS and before forming somites (Chapman et al., 1996). Interestingly, in the absence of Tbx6 expression cell which normally would develop to presomitic mesoderm were in turn converted to neural tissue derivatives, due to the antagonistic expression of *Sox2* taking over (Takemoto et al., 2011). Associated with *Tbx6* is *Msgn1*, which is expressed from gastrulation to somite formation in cells of the paraxial mesoderm. Its expression has been shown to directly affect *Tbx6* expression, and thus, the fate specification of presomitic mesoderm (Chalamalasetty et al., 2014; Yoon & Wold, 2000). Other essential genes for the further differentiation and specification of the paraxial mesoderm in these early stages include *Paraxis, Pax3, Foxc1/2, Mesp2* and *Meox1*/2 (Kume et al., 2001; Mankoo et al., 2003; Saga et al., 1997). Meox1 and Meox2 are involved in both sclerotome and dermomyotome development, and can compensate for each other (Mankoo et al., 2003). On the other hand, *Pax3* is downregulated in the specification of sclerotome, but its expression is preserved in the dermomyotome (Cairns et al., 2008), where it is involved in the early muscle development (Tajbakhsh et al., 1997). The mesenchymal tissue of the sclerotome expresses other key regulators such *Pax1*/9, *Sox9* and *Nkx3*.2 (Christ et al., 2007). *Pax1* is paramount for the axial skeleton formation (Wilm et al., 1998) and *Pax9* for the skeletal formation of the limbs and the skull (Peters et al., 1998). *Nkx3*.2 is downstream of *Pax1* and *Pax9*, and it has a pivotal role in chondrogenesis and vertebral development (Herbrand et al., 2002).

The correct gradient of FGF/Wnt with the influence of RA is needed for paraxial mesoderm patterning and the early steps of somitogenesis (**Figure.6**) (Aulehla & Pourquié, 2010). Wnt signalling from the roof plate and surface ectoderm will continue to be necessary for the maintenance of the somite epithelial stand and to form the dermomyotome, while it creates a gradient with the SHH signalling which is secreted from the notochord and the floor plate (Cairns et al., 2008; McMahon et al., 1998). Moreover, for the proper development of sclerotome, exposure to low levels of Wnt/BMP signalling and higher levels of SHH mediates the fate of the cells (**Figure.6**) (Cairns et al., 2008; McMahon et al., 1998). Indeed, SHH induces the expression of *Pax1/9* and *Sox9* through *Gli2/3*, and mice lacking proper SHH signalling have severe phenotypes on sclerotome derived tissues (Buttitta et al., 2003; Chiang et al., 1996).

1.3.b.ii. *Intermediate Mesoderm.*

Following up the paraxial mesoderm in a more lateral direction, is the intermediate mesoderm (IM), which is presented as a narrow line of tissue between paraxial and lateral plate mesoderm (Saxén & Sariola, 1987). Inhibitory pathways from paraxial and lateral plate mesoderm, further governs these tight boundaries (R. G. James & Schultheiss, 2005; Wilm et al., 2004). IM is further subdivided in an anteroposterior manner to pronephros, mesonephros and metanephros (Sariola & Sainio, 1997). Pronephros and the nephric duct, which comes from the most anterior part of the IM, are the first structure to arise as distinct tissues (Mauch et al., 2000; Obara-Ishihara et al., 1999; Vize et al., 1997). Interestingly, IM has been suggested to harbour paraxial progenitors during the early exit from PS, due to the fate duality of some cell populations (Cambray & Wilson, 2007). This may be one more case of the continues effect of BMP signalling from posterior PS and lateral plate mesoderm, where it influences the dynamic fate of mesodermal cells (**Figure.6**) (Fujiwara et al., 2002; Miura et al., 2006;

Winnier et al., 1995). Furthermore, absence of fated paraxial mesodermal cells key transcriptional factors, causes the disturbance of the normal IM development, but not of the lateral plate (Wilm et al., 2004). *Lim-1/ Lhx1* is one of the first key genes expressed in IM, where in its absence no nephrotic tissue is being developed (Carroll et al., 1999; Carroll & Vize, 1999; Shawlot & Behringer, 1995). At around mouse E8.5, markers of IM fated cells *Pax2/8, Gata3* and *Osr1* will begin to express and will mark the early plastic population of IM cells (Bouchard et al., 2002; Marcotte et al., 2014; Q. Wang et al., 2005). *Pax2* and *Lim2* will mark the anterior IM (A.IM) and drive the cells to pronephros (Barak et al., 2005; Bouchard et al., 2002), while *Eya1*, followed by a later enriched expression of *Osr1* and *Pax2*, marks the more posteriorized cells of the IM that will contribute to the metanephric structures (Gong et al., 2007; R. G. James et al., 2006; Sajithlal et al., 2005). Meanwhile, it has been shown that *Pax2*/8 act upstream of *Lim-1* and *Gata3*, and they establish a regulatory network for the further morphogenesis for the IM derived nephrotic tissues (Boualia et al., 2013; Grote et al., 2006). Additional posterior IM markers include *Gdf11* and *Hoxd11* (Izpisúa-Belmonte et al., 1991; McPherron et al., 1999), where *Hoxd11* has been speculated to mark the latest populations of IM arising from the PS (Taguchi et al., 2014). In parallel with the fating of posterior IM, at around E9.0 of mouse, the first structures of nephric duct from the A.IM are emerging, and start to migrate and expand caudally, with a mix population of leading mesenchymal cells followed by epithelial ones (Chia et al., 2011; Soofi et al., 2012). Following up, the *Hoxd11+/Pax2+/Eya1+* cell population will be incorporated to the elongating nephrogenic cord, and they will initiate the topical expression of Gndf (Barak et al., 2012; P. P. L. Tam & Beddington, 1987).

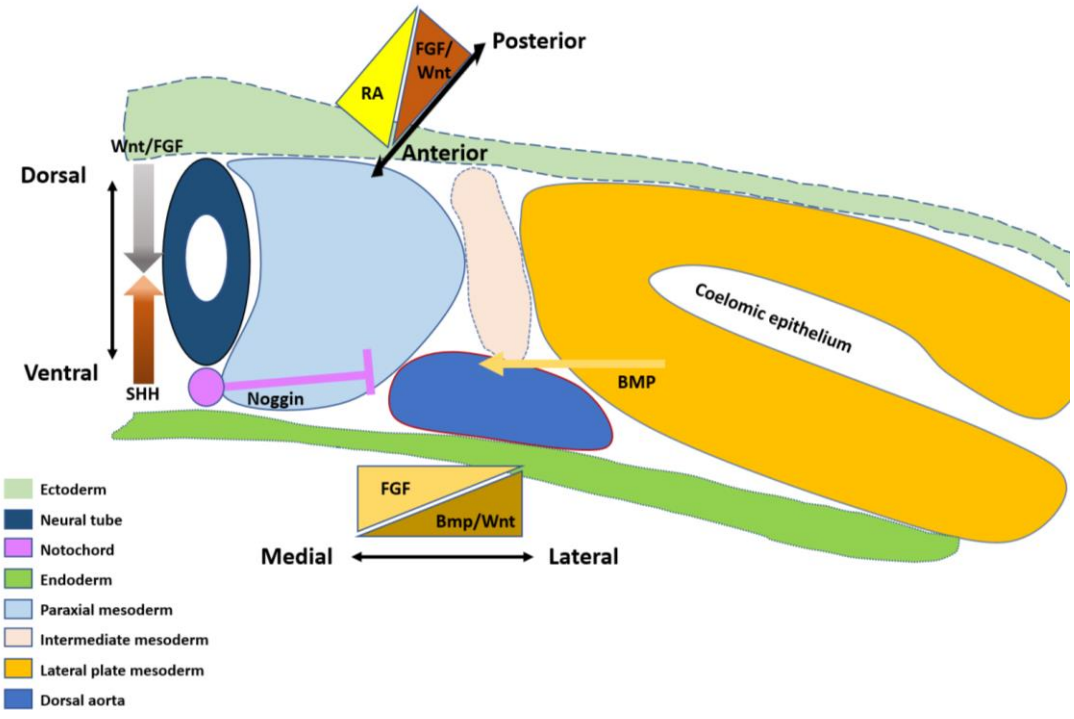


Figure.6. Abstract representation of mouse around E9 transverse section and the crosstalk of signalling pathways. From anterior part of the embryo comes a RA pulse patterning with the posterior originated FGF/Wnt signalling. FGF/Wnt also forms a gradient from dorsal to ventral, where it antagonises the SHH signalling coming from the notochord and endodermal tissues of the gut. Notochord also produces Noggin, which inhibits the effect of the lateral BMP signalling. From lateral to mediuml there is also an BMP/Wnt signalling gradient, opposite to the FGF one.

The fating of anterior-posterior IM cells is determined by the overlapping signalling ques from the surrounding tissues. In chicken, Activin/Nodal signalling from the dorsal neural tube affects this patterning (Barak et al., 2005; Preger-Ben Noon et al., 2009). Following the medial-lateral patterning, the IM needs low levels of BMP signalling (R. G. James & Schultheiss, 2005), with the addition of Wnt and FGF signalling; the correct gradient balance between these three signalling pathways governs the formation of the initial IM (Aulehla et al., 2003; Dubrulle & Pourquié, 2004; Naylor et al., 2017; Ueno et al., 2007; Warga et al., 2013). Another competing gradient to Wnt and FGF signalling is RA (**Figure.6**), which at this point of development is produced by anterior lateral plate and presomitic mesoderm (Niederreither et al., 1997, 1999). RA is a promising pathway for the development of IM through its interplay with paraxial mesoderm encoded genes (J. Li et al., 2015; Wilm et al., 2004), impairment of the RA pathway in mice leads to delayed IM formation, but not absence of it (Cartry et al., 2006). Furthermore, in Xenopus it has been demonstrated that Activin/RA can promote the formation of pronephros (Moriya et al., 1993), while in chick embryo RA can induce the formation of A.IM with Activin/Nodal signalling (Preger-Ben Noon et al., 2009). RA can potentially affect the patterning and the specification of IM similarly with somitogenesis (Diez del Corral & Storey, 2004), as *in vitro* experiments has shown that RA can induce IM fate (Araoka et al., 2014; Oeda et al., 2013).

1.3.b.iii. *Lateral plate mesoderm.*

Lateral plate mesoderm (LPM) is the third compartment of the mesoderm, and it is suspected to be evolutionary the oldest, by having common ties with the mesendoderm (Bertrand & Escriva, 2011; Holland, 2018; Kozmik et al., 2001; Onimaru et al., 2011) and its formation being well conserved (Prummel et al., 2019) . The progenitors of LPM can be found in large bilateral stripes across the A-P axis in the lateral part from each side of the notochord following up the gastrulation (Kessler & Melton, 1994; McDole et al., 2018; Prummel et al., 2019). There is no bona fide description of LPM, thus post-gastrulation marker genes for this tissue have been described, including, *Foxf1, Bmp4, Hoxb6, Hand1, Hand2, Gata4, Osr1* and *Prrx1* (Becker et al., 1996; Firulli et al., 1998; J. F. Martin & Olson, 2000; Ormestad et al., 2004; Prummel et al., 2019; Rojas et al., 2005). T does not mark any population of LPM progenitors after exiting PS, in contrast with the other mesodermal lineages, and its deletion does not affect the further development of the tissue and its products (Clements et al., 1996; K. M. Loh et al., 2016; Wilkinson et al., 1990). LPM, as we described so far, it characterized by its high Bmp expression, but it is specified also from the high ventral BMP signalling (**Figure.6**) (Ferretti & Hadjantonakis, 2019; Nishimatsu & Thomsen, 1998). BMP has an immediate effect on the fate of the LPM derived tissues (Hammerschmidt et al., 1996; Mullins et al., 1996; Sidi et al., 2003)and promotes the LPM development in the expense of paraxial mesoderm (R. G. James & Schultheiss, 2005; Pourquié et al., 1996; Tonegawa et al., 1997). Indeed, impairment of the BMP signalling on LPM causes a shift in the normal patterning, due to the disruption of the FGF/BMP gradient and paraxial mesoderm takes over (B. Ciruna & Rossant, 2001; Miura et al., 2006; Yamaguchi et al., 1994). So far, it is understandable that in all tissues there is an interplay of signalling pathways, and LPM is no exception, having influences from BMP, FGF, RA and canonical Wnt pathway for its proper formation and specification (**Figure.6**) (Fürthauer et al., 2004; Lescroart et al., 2018; Rossant & Tam, 2009; P.-F. Xu et al., 2014). These signalling ques will also position LPM properly in relation with adjusted tissues (Erter et al., 2001; Heisenberg & Solnica-Krezel, 2008; Saykali et al., 2018), and will later segregate the LPM in dorsal somatic layer and a ventral splanchnopleuric layer, while expanding on the A-P axis and forming different patterns.

Deriving from the LPM, heart progenitors are among the initially specified mesodermal cells directly after their exit from PS around mouse E7.0. They come in two waves, and they migrate anteriorly to form and develop the heart tube (Devine et al., 2014). Single cell RNA sequencing has confirmed this patter, by pinpointing already committed populations of cells in PS toward cardiac fate (Lescroart et al., 2018). These data also highlighted the role of *Mesp1* in cardiac progenitor EMT, migration, and fating (Lescroart et al., 2018; Saga et al., 2000). *Mesp1* marks the earliest cardiac progenitors emerging from the mesoderm, where its expression can be traced back to the PS (Costello et al., 2011; Kitajima et al., 2000; Saga et al., 1999, 2000), and acts upstream of *Gata4, Nkx2.5* and *Hand2* (Bondue et al., 2008). Signalling from platelet-derived growth factor (PDGF) and Robo-Slit will further fate and drive the cardiac progenitors through their morphogenesis (Fish et al., 2011; Qiu et al., 2016; J. Zhao & Mommersteeg, 2018).

At the time of heart formation, the endothelium and blood tissue first develop also. They seemingly share progenitor cells and through their synchronised development they have common marker genes, but they are easily distinct to each other (K. Choi et al., 1998; Orkin & Zon, 2008; Sabin, 2002; Vogeli et al., 2006). Although, their early development is heavily linked, during their further maturation discrimination can be made, with definitive hematopoietic stem cell (HSC) expressing *Runx1* (Boisset et al., 2010; Zovein et al., 2008) and *Vegfr2* and *Flk1* are related with the expanding endothelium (Chung et al., 2002; Yamashita et al., 2000). *Runx1* induces the haematopoiesis (Zovein et al., 2008), and the VEGF (vascular-endothelial growth factor) receptor genes of *Vegfr2* and *Flk1* are driving the endothelial differentiation through the VEGF signalling (Atkins et al., 2011; K. M. Loh et al., 2016; Marcelo et al., 2013; Simons et al., 2016; Yamashita et al., 2000).

The coelomic epithelium (CE), which cover the body cavities and the organs within it, comes also from LPM (Ariza et al., 2016b; A. W. Moore et al., 1998; Mutsaers & Wilkosz, 2007), and serves as a source of mesenchymal cells (Que et al., 2008). CE has been described to contribute to a wide range of tissues including the urogenital system, liver, pancreas, spleen, smooth muscles surrounding the gastrointestinal and respiratory tract, and the vascular system (Ariza et al., 2018; Asahina et al., 2011; Bohnenpoll et al., 2013; Cano et al., 2013; Carmona et al., 2013; Chau et al., 2011; Hatano et al., 1996a; Ijpenberg et al., 2007; C. Liu et al., 2015; Que et al., 2008). More notably, *Wt1*-positive cells from the CE concentrate to form the spleen (Burn et al., 2008; Endo et al., 2015), mesenchymal cells of CE directly contribute to the outer layer of the liver (Pérez-Pomares et al., 2004), while indirectly contributes to the pancreatic development (Deutsch et al., 2001; M. Kumar et al., 2003), and a thickening of the CE near the IM will give rise to the AGP (Hatano et al., 1996a; Ikeda et al., 1994). From the CE till the genital ridges the expression of *Osr1*, *Raldh2*, and *Wt1* is apparent (Bohnenpoll et al., 2013), and *Gata4*, *Tbx18*, *Tcf21* and *Wt1* can stated as the earliest markers of the genital ridge mesenchyme (Airik et al., 2006; Cui et al., 2004; Häfner et al., 2015; Hammes et al., 2001; Y.-C. Hu et al., 2013; Karl & Capel, 1998). Migratory mesenchymal cells from the CE to the GP are labelled by *Emx2* expression, which also has a key role in this event (Kusaka et al., 2010). Furthermore, CE is interconnected with the development of the reproductive tracts. It has an active contribution on the Mullerian duct (Guioli et al., 2007), while the Wolffian duct provides stability to the CE and it is not influenced by it (Shaw & Renfree, 2014; Yoshino et al., 2014). Lastly, it is important to remember that CE is also a signalling hub, which affects the surrounding and distal tissues, by expressing molecules such as FGF9, TGFb3 and RA (Bragg et al., 2001; Colvin et al., 2001; Dickman et al., 1997; Malpel et al., 2000; A. C. White et al., 2006).

1.4. Extracellular matrix.

The extracellular matrix (ECM) is the non-cellular component, cell/tissue-specific environment in which the cells exist, and each is composed of an array of multidomain macromolecules uniquely organized to fit the requirements of the given niche. Each ECM is tissue-specific, in terms of their physical, topological, and biochemical composition. This specificity is formed dynamically during tissue development through the biochemical and biophysical crosstalk between the different cellular/tissue components and the ever evolving cellular and protein microenvironment of the given tissue (Frantz et al., 2010). The most apparent role of the ECM is to create the basic scaffold where a tissue can exist, where firstly from a mechanical point those macromolecules are linked together and give to the tissue its mechanical properties by forming a structurally stable complex. Secondly the ECM acts as a biochemical niche by having a reservoir of growth factors and bioactive molecules, further contributing to the unique properties of each tissue. Those attributes of the ECM makes it a focal point for determining and controlling the homeostasis and the most basic, but also more advanced, behaviours and properties of cells such as proliferation, adhesion, migration, polarity, differentiation, and apoptosis. Thus, in order to understand better a cell/tissue function in an organism, it is important to take in consideration the whole niche of it, which includes the ECM (Ginsberg et al., 2005; Lu et al., 2011; Mecham, 2012; Padhi & Nain, 2020). Key components of the ECM are fibrous proteins and proteoglycans (Järveläinen et al., 2009; Schaefer & Schaefer, 2010).

The effect of the ECM can be partially attributed to its heterodimeric transmembrane receptors, the integrins. Integrins, which consist of dimers with a and b subunits of it, interact with extracellular matrix glycoproteins/ligands through different binding sites, and translate the ECM signalling by the recruitment of multiple cytoskeleton-associated proteins to regulate intracellular signalling pathways affecting a plethora of cell processes (Giancotti & Ruoslahti, 1999; Ginsberg et al., 2005; Hynes, 2002; Meredith et al., 1996).

A paramount motif for the cell adhesion, the RGD, has been first identified first in FN, and since then it has been found in multiple other ECM proteins like Collagen, Vitronectin, Laminin and Tenascin. The interaction between the RGD motives and the integrin receptors modulates the adhesion, and its mutation causes drastic loss of cell-adhesive ability (Obara et al., 1988; Pierschbacher & Ruoslahti, 1984). The absence of this motif showcased the great impact of it during the development and the importance of cell adhesion, where mutated mice died by embryonic day 10, with multiple deformities and defects in the vascular system (Obara et al., 1988; S. Takahashi et al., 2007).

1.4.a. Fibronectin.

Fibronectin (FN) is a high molecular weight dimeric with flexible strands glycoprotein where the two similar subunits are linked through a pair of disulphide bonds near their C-terminus 68. Fibronectin is widely expressed by a variety of cells in embryonic and adult tissues. There are three types of repeating modules in each fibronectin subunit. There are 12 type I modules, which are present uniquely in chordate (Tucker & Chiquet-Ehrismann, 2009). The type II modules are rarer, where each subunit has 2 of them, but they are present in a wider range of organisms, including non-vertebra ones (Ozhogina et al., 2001) . The type III module (termed FN3) has 15–17 repeats in a fibronectin subunit, it is highly evolutionary conserved and multiple modules of it has been sighted in other ECM, receptor, and cytoskeletal proteins (Bork & Doolittle, 1992). Those three modules consist the 90% of the fibronectin sequence (J. Xu & Mosher, 2011).

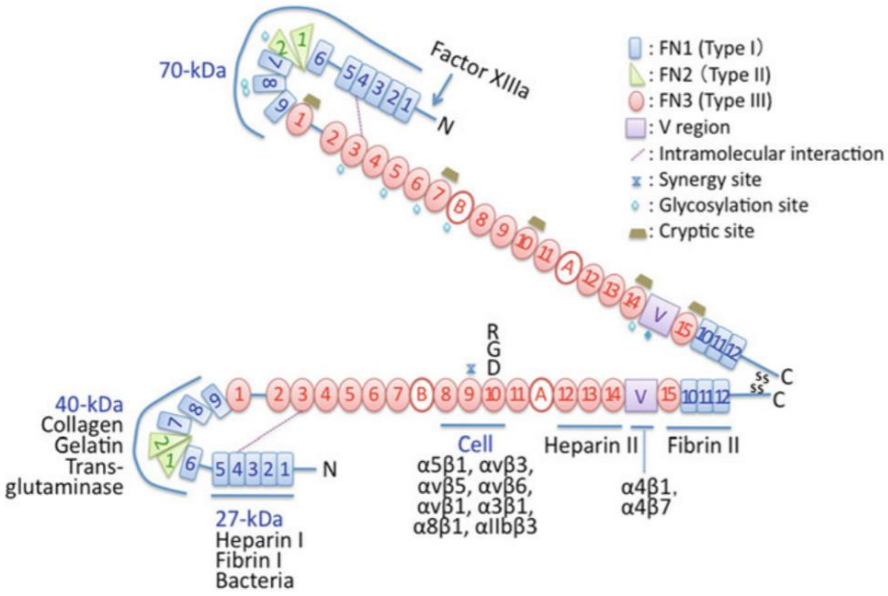


Figure.7. From (J. Xu & Mosher, 2011) Molecular structure of fibronectin. Representing the two monomers of fibronectin linked at their C-terminus by a pair of disulphide bonds. Depicting the type I modules (blue rectangles) termed FN1, type II modules (green triangles) termed FN2, and type III modules (salmon ovals) termed FN3. The number of FN3 modules varies based on alternative splicing. The alternatively spliced V region is shown as a purple square. Proteolytic 27-kDa, 40-kDa, and 70-kDa N-terminal fragments and the protein-binding sites on fibronectin are underlined with receptors listed.

FN is divided into plasma and cellular fibronectin. Plasma FN is synthesized by the hepatocytes in the liver, and it is found in blood plasma in soluble form, normally around 300 mg/ml. Plasma FN participates in thrombosis. This event is modulated by Fibronectin–Fibrin interaction, though is very weak at normal temperature, a crosslinking of Fibrin and Fibronectin is mediated, and it stabilizes this interaction, and it incorporates Fibronectin into the fibrinclot. This event can stimulate cell adhesion or migration into Fibronectin–Fibrin clots upon wound healing, a crucial mechanism for injury recovery and homeostasis (Ni et al., 2003). Cellular fibronectin, which is secreted by cells and directly contributes to the insoluble ECM, is organized in fibrils (George et al., 1993). The assembled FN fibrils exist as one of the primordial and crucial for the proper embryonic development components of an ECM; there other FN-interacting proteins can harbour and provide necessary support for the creation of the tissue's ECM (Darribere et al., 1990; Hynes, 2009). Fibronectin fibrillogenesis cannot take place in normal tissue conditions without the presence of assembly competent cells; this seems to be the case for both types of FN (Bae et al., 2004).

This fibrillar network has a leading role in the regulation of cell functions through interaction with cell surface receptors (integrins); where FN specifically interacts with other proteins, including other ECM proteins (Yamada et al., 1980). Furthermore, the FN-integrin interaction promotes the unfolding of cryptic fibronectin, which is paramount for fibronectin matrix assembly (Geiger et al., 2001). Through those interactions and its matrix assembly, FN has been demonstrated to be crucial part of cell migration and adhesion, and tissue development, differentiation, and morphogenesis (Dzamba et al., 2009; X. Zhou et al., 2008); complete depletion of the fibronectin gene is lethal at embryonic day 8.5 (George et al., 1993). FN matrix can also create actin microfilament bundles by the co-operating binding of heparan sulphate proteoglycans through the heparin-binding domains of it, and by cell-to-cell communication with the cell-binding domains. Thus, further enhancing its role in the niche of the

given tissue (Izzard et al., 1986; Lark et al., 1985; Laterra, Culp, et al., 1983; Laterra, Silbert, et al., 1983; Woods et al., 1986). It is apparent that FN acts as a scaffold and modulates the activity and effect of several growth factors directly or indirectly, on the surrounding cells and tissues; given their proper environment (Frantz et al., 2010; Hynes, 2009).

1.4.b. Collagen.

Collagen is an insoluble fibrous protein with overrepresentation throughout the animal species in ECM and connective tissue. It is secreted by fibroblasts and epithelial cells, and it gets incorporated to the ECM. There are 28 different types of collagens in vertebrates, where each collagen has 3 alpha chains forming a triple helix (Ricard-Blum, 2011). The different types of collagens can then be subdivided to 3 categories, according to their properties: network-forming, fibril-forming, fibril-associated (FACITs) (Yue, 2014).

Collagens can assemble into fibrils by themselves (G. C. Wood, 1964) and form ECMs, or they can also incorporate other proteins. Notably, a great interest has been shown for the collagen and FN interaction, where FN has been shown to interact with collagen fibrils (Kleinman et al., 1981). Indeed, native collagen can bind with FN, due to protein unfolding which occurs locally because of the body temperature (Leikina et al., 2002). Further supporting these findings, initial studies have shown that denatured collagen has even greater affinity in binding FN than the native form (Engvall et al., 1978). The creation of such an ECM network is pivotal for adhesion, migration, and homeostasis of the tissue in question (Pankov & Yamada, 2002); as well as during development, where other ECM proteins contribute to further evolve the niche (Padhi & Nain, 2020).

1.4.c. Laminin.

Laminin is another ECM protein evolutionary conserved through the species and it can be traced from very early stages of embryonic development 94,95. Laminins are large heterotrimeric glycoproteins between 400 to 900 kDa, where each unique subunit is transcribed by different gene. Each subunit is a polypeptide chain, alpha, beta, or gamma, with 5 alpha, 3 beta and 3 gamma chains have been studied in 16 laminin variants 96. The laminin variants are a result of a combination of these three alternately assembled subunits. Laminins, by mixing and interacting with themselves, and by also self-polymerizing, they create filaments and layered sheets. These structures compose the majority of the basal lamina and trigger the formation of the basement membrane, where they also have a paramount role (S. Li et al., 2002; Rennard & Martin, 1979; Schéele et al., 2007).

Laminins have a major role in the adhesive role of the basement membrane with the surrounding cell layers, predominantly through the integrins (Belkin & Stepp, 2000; Yurchenco & Wadsworth, 2004). These interactions have focal role also to cell migration, proliferation, differentiation, and survival, by activating cascades of intracellular signalling (Belkin & Stepp, 2000; Colucci et al., 1996; Givant-Horwitz et al., 2004; Gonzales et al., 1999; Hintermann & Quaranta, 2004). Additional interactions with collages, heparan sulfate proteoglycans and other components found in ECMs, and ECM networks (eg Collagen 4 network), are important for tissue development and morphogenesis (Mayer et al., 1998; Miner & Yurchenco, 2004; Padhi & Nain, 2020; Rifes & Thorsteinsdóttir, 2012; Yurchenco & Schittny, 1990).

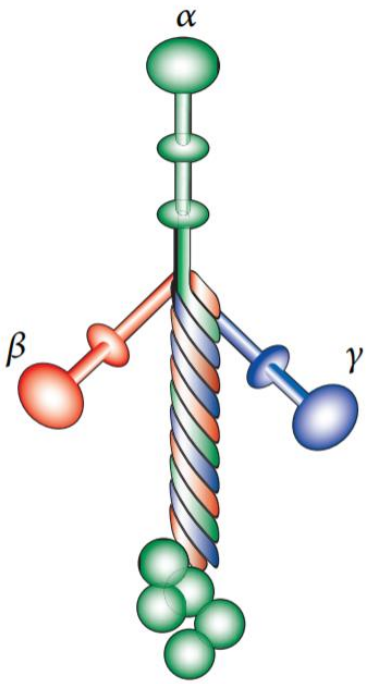


Figure.8. (Plantman, 2013) schematic drawing of heterotrimeric laminin structure composing of one alpha, one beta and one gamma chain, where all three subunits interwire at a point and form a coiled-coil structure with a series of five globular domains in the α chain C-terminal.

1.4.d. Tenascin.

Tenascins (TN) are large hexametric extracellular matrix glycoproteins with each subunit consisting of a linear array of structural domains (Figure). There are four members of the TN family; TN-C, R, W, X110. Tenascin-C was the first and best described TN. It is found around migrating cells in the embryo, at sites of epithelial–mesenchymal interactions and of branching morphogenesis, and in developing connective tissues. TNC persists in some connective tissue in the adult, at sites of tissue repair and regeneration, and in the stroma of many tumours (Chiquet-Ehrismann & Chiquet, 2003; Jones & Jones, 2000; Padhi & Nain, 2020). TNR expression is in the central and peripheral nervous system (Rathjen et al., 1991) and TNW mainly in mesodermal derived tissues (Scherberich et al., 2004). TNX is primarily expressed in connective tissues like dermis, epimysium, and blood vessels both during development and in the adult (Bristow et al., 2005).

TN, and predominately TNC, has been labelled as an anti-adherent protein and interestingly, it has been described to bind with FN to fine tune adhesion and mobility, during development and tumorigenesis (Jones & Jones, 2000; Trebaul et al., 2007). This can be seen by the comparison of the cell morphology with cells culture with and without TNC; most likely due to the lack of the rearrangement of the actin cytoskeleton, compared to more adherent ECM proteins like FN (Ambort et al., 2010; Lotz et al., 1989; Sriramarao et al., 1993). Indeed, TN has a direct impact on ECM properties which directly affect the residual cells, where sometimes coming to oppose the effect of FN (Midwood & Schwarzbauer, 2002; Murphy-Ullrich et al., 1991; Pesheva et al., 1994; Tucker et al., 2001).

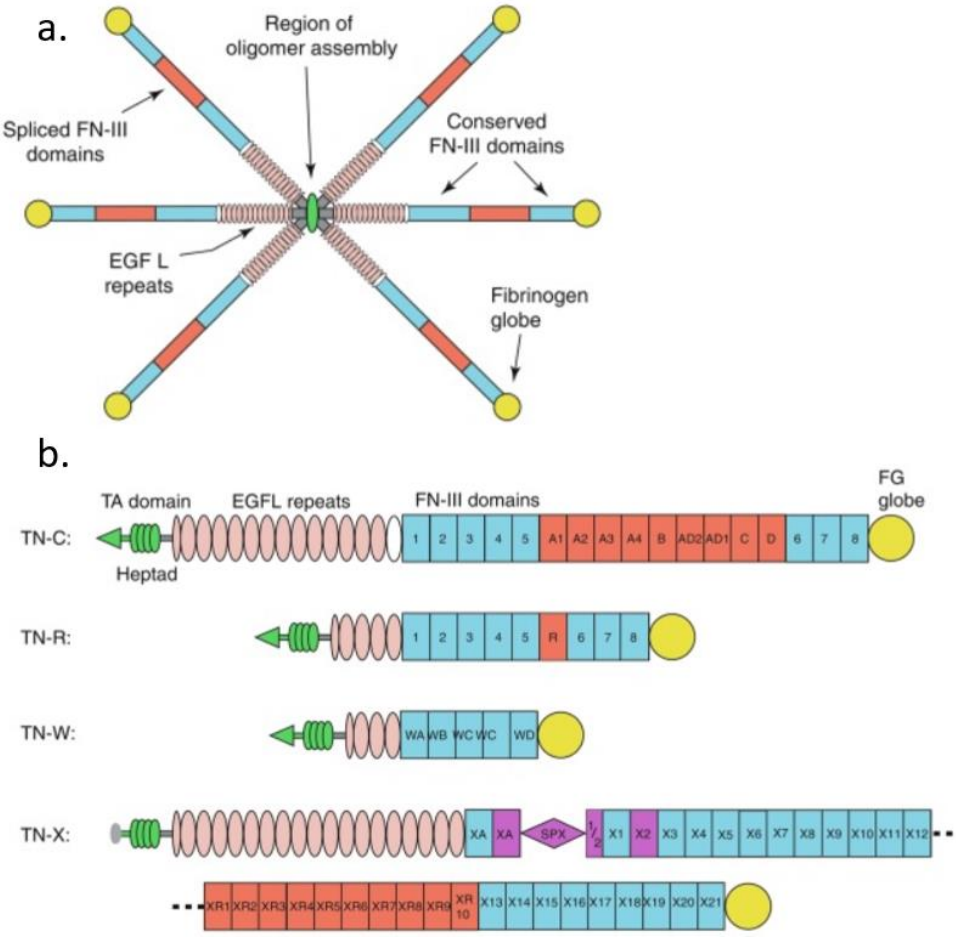


Figure.9. (Nakamura & Bornstein, 2020) Representation of assembled Tenascin, following the same depiction as (b). (b) Structural models of the four members of the tenascin family. Green for the TN assembly (TA) domain. TN-X has a gray oval indicating that the cysteines required to link trimers in higher order multimers are missing. The EGF-like repeats are common to all vertebrates (salmon oval). In the case of TN-C, an additional repeat (white oval) is present. There are several categories of FN-III repeats (blue, red, or purple rectangles) according to their composition or origin during evolution. Extra serine- and proline-rich domain (purple diamond) is found in TN-X. A fibrinogen globe (yellow circle) in the end of each macromolecule is common to all TN.

1.4.e. ECM in developing adrenal gland.

ECM is a crucial component for the physiological development of many tissues, and their later homeostasis; and the adrenal gland is no exception (Chou et al., 2013; Feige et al., 1998). Although their distribution differs based on the organism, both in fetal and adult adrenal gland (Chamoux et al., 2001; Feige et al., 1998; Otis et al., 2007; Pellerin et al., 1997).Distinct localization of ECM proteins through the fetal human adrenal gland has been observed. More specifically three main ECM proteins have been mapped, Collagen IV, fibronectin, and laminin, together with the expression pattern of five integrin subunits β1-2 and α1-3. Collagen IV has been shown to have a broad pattern in the fetal cortex, while laminin and FN are having a gradient effect where laminin defines the DZ and FN the FZ, while both can be found in the transitional zone. Notably, the capsule exhibits all 3 of the ECM components that were mentioned, with stronger labelling of Collagen IV. Moreover, a3 integrin subunit has been localized within the FZ and b2 on chromaffin cells. Capsule is also labelled for all integrin subunits, which are found in the rest fetal adrenal (**Figure.10**)(Chamoux et al., 2001; Virtanen et al., 2003). Based on this combination of ECM proteins and receptors, it is possible that Collagen 4 and FN promote migratory effects in the adrenal gland (Chavakis et al., 2005; Gotwals et al., 1996).

Particularly, the integrin receptor α3β1 has been identified as a moderate to weak affinity for FN (Kühn & Eble, 1994), this strengthens the opinion of the free migration of cells in the fetal adrenal gland (Freedman et al., 2013; Ishimoto & Jaffe, 2011; Mitani et al., 1999, 2003). In addition, TNX, which also potentially counteracts the adhesiveness of FN, has an adrenal-specific variant and it has been linked with CAH-X syndrome (Miller & Merke, 2018), possibly due its gene overlapping with the one of 21-hydroxylase (Tee et al., 1995).

ECM has a direct effect on function, hormonal production, and the morphology of human fetal adrenal disassociated cells from the different zones; as it has been already shown *in vitro,* when compared with seeding cells on plastic (Chamoux, 2005; Chamoux et al., 2002). In line with their topology, laminin is favouring cells with characteristics of DZ such as round and more proliferative morphology, clustering, and reduced in size (Chamoux et al., 2002; Fujieda et al., 1981), and actin distribution of the DZ (Chamoux et al., 1998). In contrast with laminin, cells growing on FN were less adherent and proliferative. This is marked by the two distinct populations of cells presented on this ECM: polygonal stereodogenically active cells and round non proliferative apoptotic ones; profiling cells of the FZ. Meanwhile the presence of Collagen IV in the cell culture increases cell proliferation, a characteristic of DZ/TZ, promotes the morphologically enlarged cells of TZ/FZ and enhances their metabolic characteristics; this ECM does not exhibit substantial amount of DZ cells (Chamoux et al., 2002). Thus, those environments *in vivo* will be responsible for laying the blueprint of the future functional zonation (Bocian-Sobkowska et al., 1993).

The importance of the ECM for the proper function of the adrenal gland continues on the adult animal, as it has been shown mainly *in vitro*, which is translatable *in vivo* (Carsia et al., 1997; Otis et al., 2007; Pellerin et al., 1997; Virtanen et al., 2003). The localisation of COL1/4, FN, and laminin, accompanied with the corresponding integrins receptors have been mapped on adult rat adrenal gland. It has been shown *in vitro* the importance of these ECMs for hormonal homeostasis and promoting proliferation; with FN and Collagen1/4 favouring more the steroid secretion and laminin the proliferation. Though, there are distinct differences in behaviour of cultured cells from zG or zF in the same matrices. Notably, zG cells form monolayers of polygonal cells, with increased ACTH responsiveness on all but laminin matrices, where they appear smaller, round, and packed together with restricted protein synthesis. zF cells have increased protein secretion on FN and Collagen1/4, and they appear morphologically smaller and clustered on FN and laminin (**Figure.10**) (Otis et al., 2007). Consequently, ECM has been proven paramount for having a close to physiological environment *in vitro* (Carsia et al., 1997) and retaining the steroidogenesis on primary cultures (Cheng & Hornsby, 1992). Thus, it can be considered also as a factor for *in vitro* differentiation experiments towards adrenocortical lineage (L. Li et al., 2019).

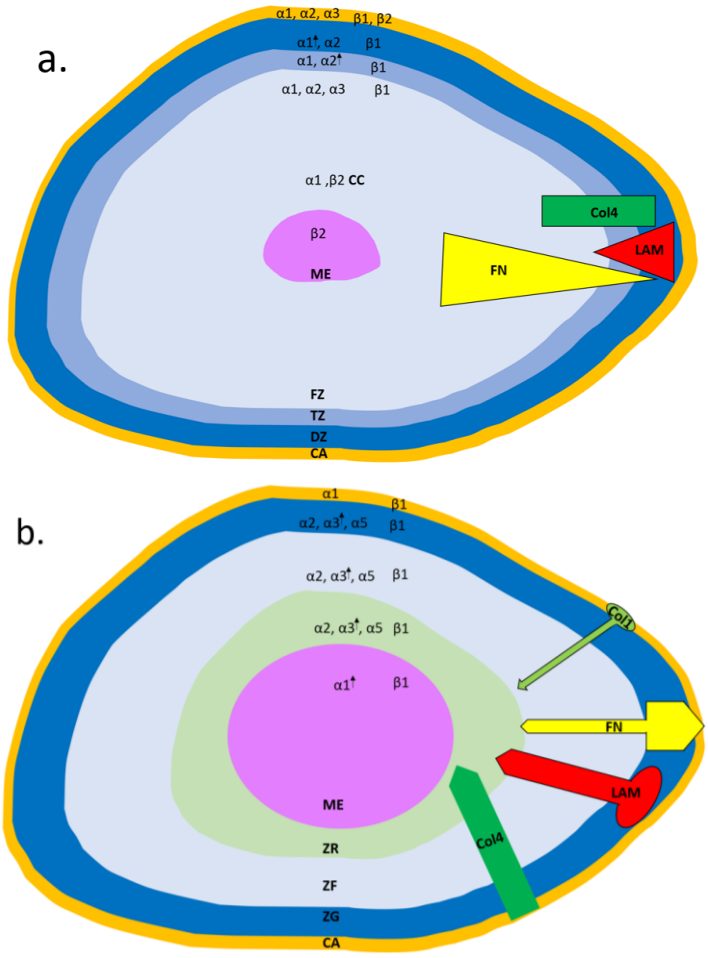


Figure.10. Depiction of the distribution of ECM proteins and their integrin receptors in the human fetal adrenal gland (**a**) and rat adult adrenal gland (**b**). **a.** Collagen4 is distributed evenly throughout the fetal gland, laminin is predominant in the DZ, and fibronectin is predominant in the FZ (Chamoux et al., 2001) **b**. Collagen1 is mainly expressed in the capsule, surrounding each glomerulosa cell and as thin fibrils in the zF. Collagen4 has moderate labelling in the capsule but, while in zF Collagen4 is presented as long thick fibrils between the radial cords of fasciculata cells. Laminin was observed primarily in zG, with short fibrils penetrating both zF and zR. FN staining is moderate in the capsule, while there is a strong presence around each glomerulosa cell and can be seen as discontinuous fibrils throughout the cortex. Except for Collagen4, other of the mentioned ECM proteins has almost no presence in zR. Furthermore, Collagen1 surrounds every CC cell, while Laminin and Collagen4 are found around clusters of CC. FN is restricted to the border of blood vessels (Otis et al., 2007).

1.5. Aim of this study.

The aim of this study is to decipher the early development of the AGP and what drives the differentiation of fetal steroidogenic progenitor cells to a more mature steroidogenic adrenocortical fate. To date published studies utilised protocols that induced adrenal steroidogenic cell differentiation by forcing the expression of NR5A1 (Ruiz-Babot et al., 2018; Tanaka et al., 2020). The cells/tissue produced by such protocols lack the full functionality or/and the proper homeostasis of the adrenocortical tissue, indicating a short viability if these cells will be used in treatments. On the other hand, this approach has been used as a steppingstone for successful protocols aiming to a gonadal fate, where they still lack functional studies (Knarston et al., 2020; Rore et al., 2021). In combination with protocols which are not using forced *Nr5a1* expression (Seol et al., 2018; Y. Yang et al., 2020) they provide us with useful information on what the steroidogenic cells may need, while at

the same time avoiding the gonadal fating of those. Our hypothesis is that more properly fated cells will be needed to acquire a full adrenocortical identity and integrate to the *in vivo* system. To achieve this, I followed a step-to-step differentiation approach based on known bibliography and preliminary *in silico* generated data on the early differentiation and specification of fetal adrenocortical cells. This approach will not only provide a robust *in vitro* differentiation protocol, but it can provide useful information about the development of the tissue during these early steps.

2. Results

2.1. Mesodermal *in vitro* differentiation protocol.

The first goal of the project was to establish a protocol that faithfully drives mESCs to a fate resembling the CE. The first steps of such a differentiation had already been established in our lab by optimizing existing protocols (Motamedi et al., unpublished). In brief, cells were induced to differentiate towards the EpiSC state through treatment with Activin-A and FGF2 in feeder free medium (Tosolini & Jouneau, 2016). The PS-stage was reached by treating cells in a defined medium for mesodermal differentiation with Chir99021, an inducer of the Wnt pathway that inhibits GSK-3 (Takasato et al., 2014, 2015). Finally, a fate close to CE cells was obtained by adapting the existing protocol for IM, to obtain a more anteriorized and lateralised (LPM) mesoderm (Oeda et al., 2013; Takasato et al., 2015). At this time a more defined protocol was published which exhibit cells having the potential that allowed differentiation towards the Sertoli cell fate (Seol et al., 2018). In their protocol they treated mESCs directly with Chir for 1-2 days, followed by treatment with RA+FGF2 for 4 days. Having this information available, a differentiation protocol was developed through trial and error as depicted in **Figure.11a**. Optimization was performed using qPCR and immunofluorescent analysis for specific molecular markers **(Figure.11b,c)**.

Differentiation was more successful when cells were synchronized before moving to the next phase of differentiation. The introduction of an intermediate step – ES differentiation into EpiSC marked by *Fgf5* upregulation - minimized colonies that were resistant to differentiation and increased the output of mesodermal cells. Also, as EpiSC state cells resemble hIPSCs, this approach may allow an easier adaptation of the protocol for the differentiation of human cells. Furthermore, the seeding of the cells had to be optimized and a seeding concentration of around $3*10^4$ cells/cm^2 (or $6*10^4$ per well of 24-well plate) was found ideal for this protocol. The most crucial part appeared to be the PS formation, in which cells are fated for A.IM/LPM-CE lineages. As summarized in **Figure.11b,c**, the transition to PS is successful and reproducible, where Brachyury (T) expression peaks at the PS state and then fades away (**Figure.11b**). This peak of expression was also visible on the protein through analysis (**Figure.12**). Two days after Chir induction more than 70% of cells expressed T protein (**Figure.12a,b,c)**, but some colonies maintained low levels of OCT4 (**Figure.12d,e,f)**.

A large amount of cell death was observed (approximately 30-50%) during the first two days of establishing the PS state. While this is normal, excessive cell death (more than ¾ of the culture) greatly impacted the end result. Proper PS differentiation leads to the upregulation of *Wt1*, *Gata4* and *Osr1*, three marker genes of the early AGP (**Figure.11b**)(Sasaki et al., 2021). We can relate the expression of *Wt1*, *Gata4* and *Foxf1* with cells having LPM fate, and *Pax2*, *Lim1* with A.IM fate **Figure.11b,c** (Sasaki et al., 2021). The resurface of *Fgf5* can be also linked to the mesodermal fate of the cells (Haub & Goldfarb, 1991). WT1 and PAX2 did not always colocalise, suggesting differently fated cells in the culture (Figure.13). Alternatively, some of the cells may represent an earlier differentiation stage or more posterior IM fate (WT1+, PAX2-) (**Figure.13**). Thus, the combination of *Wt1*, *Osr1*, *Gata4*, *Prrx1* and *Foxf1* indicates the presence of LPM stage, and *Wt1*, *Osr1*, *Pax2* and Lim1 of A.IM population. Analysis of the endodermal markers *Foxa2* (early) and *Pdx1* (advanced) (Micallef et al., 2005), showed no specific upregulation (**Figure.11c**). However, with this protocol we observed significant upregulation of *Sox17*, a marker of both endothelial and epithelial cells derived from mesoderm and

endoderm respectively (E. Choi et al., 2012) Co-staining of cultures with the markers PAX2 and SOX17 (**Figure.14**) or LIM1 and SOX17 (**Figure.15**) revealed that rare cells co-stained for the A.IM markers with SOX17. This co-staining of LIM1-SOX17 can mean some contamination of resistant anterior PS – endoderm progenitors (Hsu et al., 2018; P. P. L. Tam et al., 2004). Alternatively, the PAX2 and LIM1 co-staining with SOX17 can imply the existence of endothelial origin, or more specifically hemogenic endothelium which is of mesodermal lineage (N. Kumar et al., 2015). Indeed, this hypothesis is likely, given additional data (see below), but a minor endoderm contamination (off target differentiation) cannot be excluded in this type of experiments. Additional experiments, in which *Sox17* was repressed by inhibition of TGFβ/Activin/NODAL pathway through chemical inhibition of the ALK4/5/7 receptors, did not yield the desired differentiation results, which may indicate the necessity of temporal SOX17 expression for this (data not shown).

Mc2r encodes the receptor for ACTH and is specifically expressed at later stages of fetal adrenal development, as well as in the adult adrenal cortex. qPCR analysis revealed upregulation of *Mc2r* after 3 days of RA + FGF2 treatment (**Figure 11.b.**). IF staining did not confirm expression of the MC2R protein in vitro (data not shown). This *Mc2r* expression can be either artifact of in vitro differentiation or it could hinder something more for the transitional stage of cells; this will be analysed and explained later.

2.2. Screening for AGP fating.

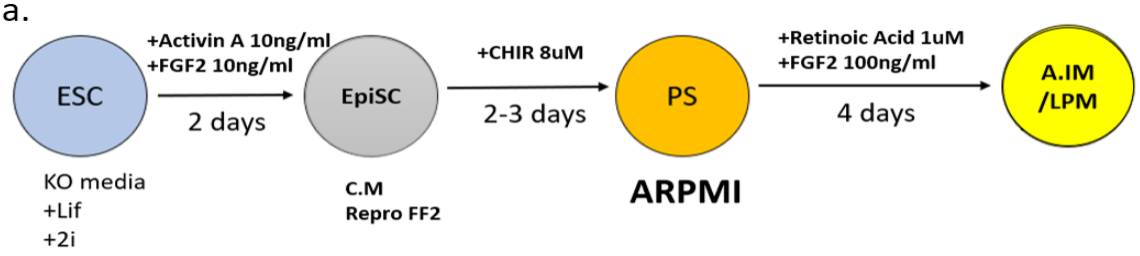
2.2.a. BMP pathway.

2.2.a.i. *BMP4 treatments of A.M.*

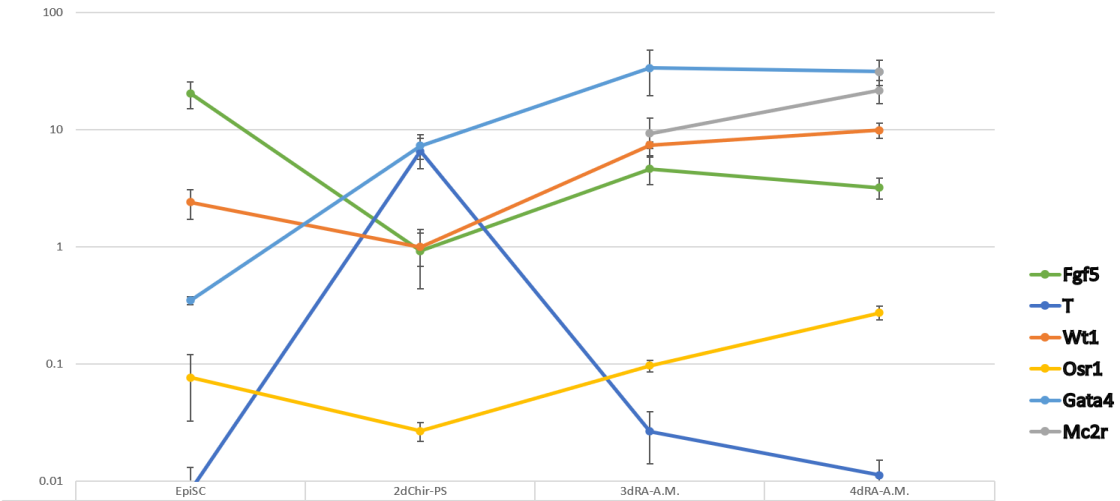
Optimizing conditions for differentiation can be improved when a visible readout is available. To this end initial differentiation experiments were carried out using an *Nr5a1-Gfp* mESCs line, a reporter that should be specifically expressed in steroidogenic cells.

BMP signalling pathway was the first to be tested by introduction of recombinant BMP4. BMP4 is a signalling molecule with important functions in mesoderm development and patterning. Indeed, laterally expressed BMP antagonises medial FGF signalling and as such patterns the embryo along the medio-lateral axis. Furthermore, BMP4 and BMP signalling has been associated with the proper regulation of the tissue (Otsuka, 2010). To test for the effect of BMP signalling in cell differentiation, AM *Nr5a1-Gfp* cells were treated with increasing concentrations of BMP4, ranging from 1 to 200 ng/ml (**Figure.16)**. GFP expression was observed after one day of treatment at all concentrations analysed. At the concentration of 50-100ng/ml the cells were healthy and expressing GFP, while at 200ng/ml cells seemed more stressed and at lower concentration there were less GFP+ cells. When the AP separates from the AGP it migrates towards the dorsal aorta, a source of BMP signalling (D. J. Anderson et al., 1997; Reissmann et al., 1996; Saito et al., 2012; Saito & Takahashi, 2015; Schneider et al., 1999; Shah et al., 1996). Therefore, an increase of *Nr5a1* expression in response to BMP4 was expected. RT-qPCR analysis, however, showed no significant upregulation of *Nr5a1* expression. Moreover, further analysis revealed a surprisingly high expression of *Nr5a1* in ESCs and EpiSCs cells (example **Figure.17g**).

a.

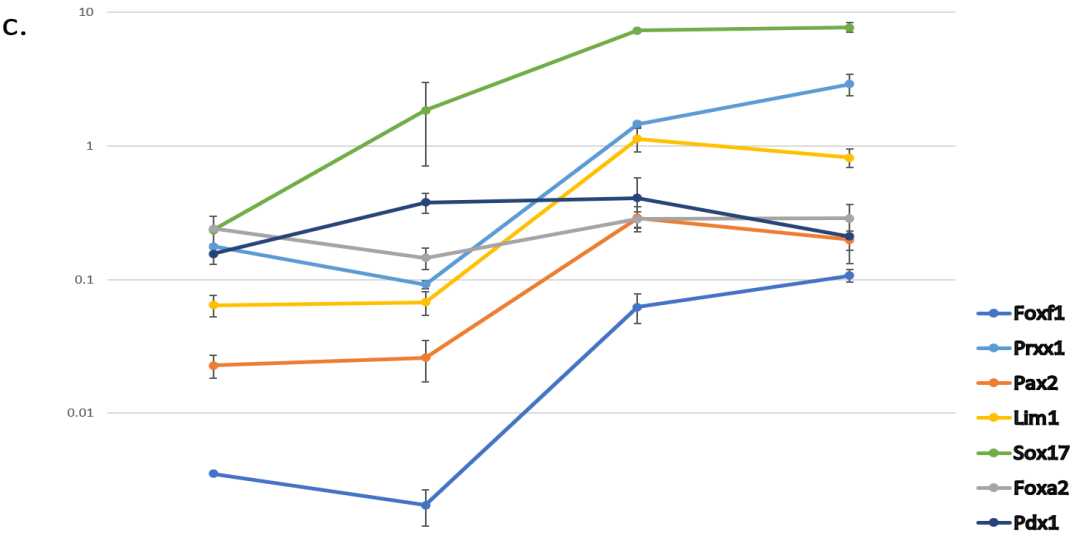


b.



	EpiSC	2dChir-PS	3dRA-A.M.	4dRA-A.M.
Fgf5	20.43945833	0.919665626	4.634866665	3.195733398
T	0.008274471	6.550674192	0.026567479	0.011203893
Wt1	2.411542626	0.998606461	7.382805819	9.9272169
Osr1	0.076483733	0.026677801	0.096553631	0.274056703
Gata4	0.346768949	7.293960925	33.76835177	31.46143943
Mc2r	0	0	9.308634462	21.6557341

c.



	EpiSC	2dChir-PS	3dRA-A.M.	4dRA-A.M.
Foxf1	0.003515638	0.002048408	0.062317789	0.107370875
Prxx1	0.176946061	0.091789782	1.460738108	2.900761312
Pax2	0.022735898	0.026026765	0.289097049	0.19855901
Lim1	0.06427505	0.067586556	1.130103908	0.816859894
Sox17	0.234310322	1.851150177	7.341361864	7.723544853
Foxa2	0.23981603	0.145077843	0.284733242	0.288069868
Pdx1	0.155684955	0.378510678	0.409383465	0.210677077

Figure.11. **a.** Schematic outline of the *in vitro* differentiation protocol to achieve a mixed population of anterior intermediate and lateral plate mesoderm. **b&c.** RT-qPCR data from key steps during the differentiation of R1, Bl6 and mix background cell lines. All results were compared with the caudal (bellow the heart, without the tail and spine) region of E9.5 of mouse embryos and normalised with Sdha expression.

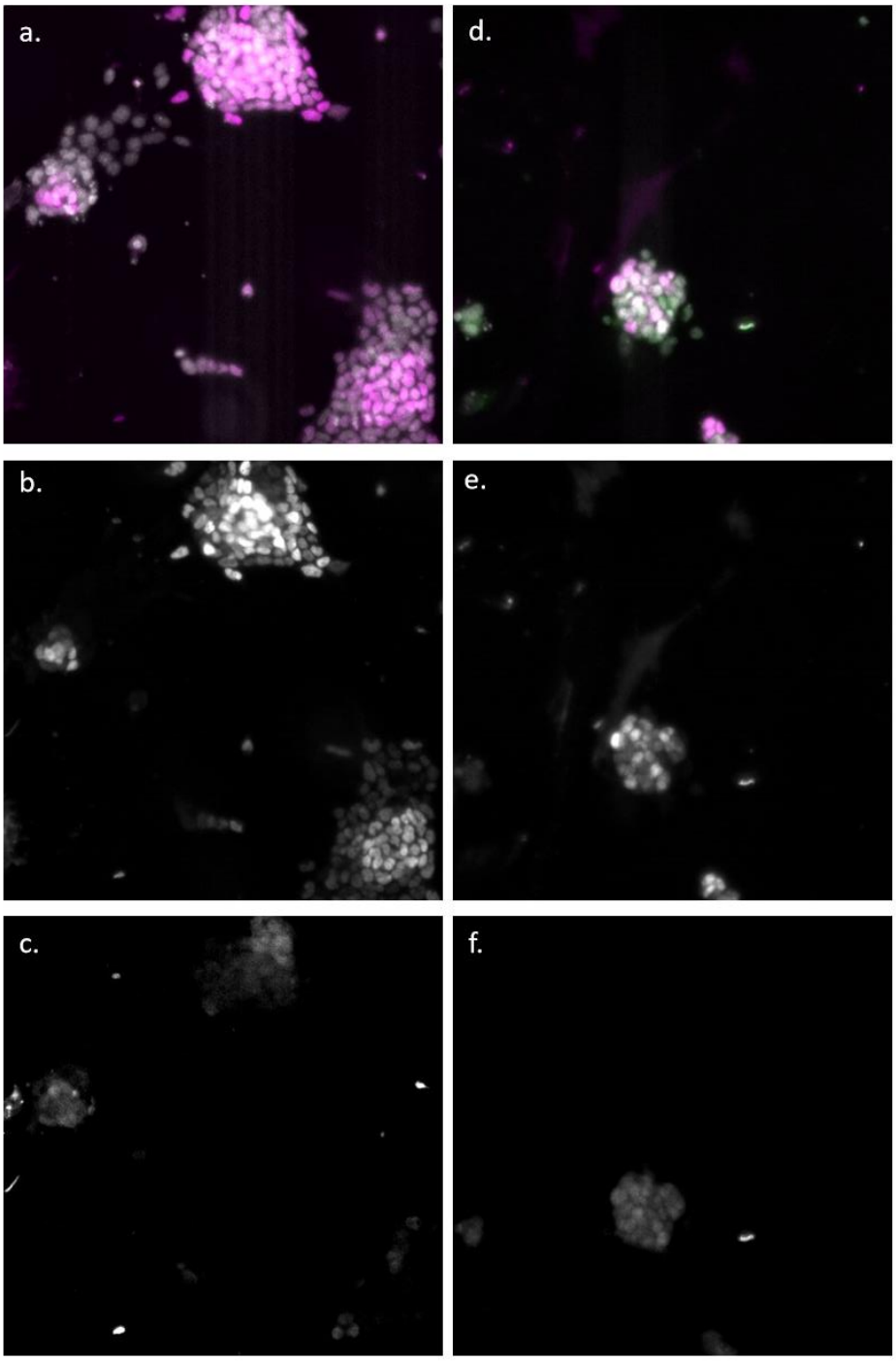


Figure.12. a & d. IF analysis after 2 days of Chir treatment for T (marker protein of PS; magenta) and OCT4 (marker protein of pluripotency; green), Hoechst was used to stain the nuclei of cells (grey). **b & e.** Individual channel for T expression. **C & f.** Individual channel for OCT4 expression. 20x magnification was used.

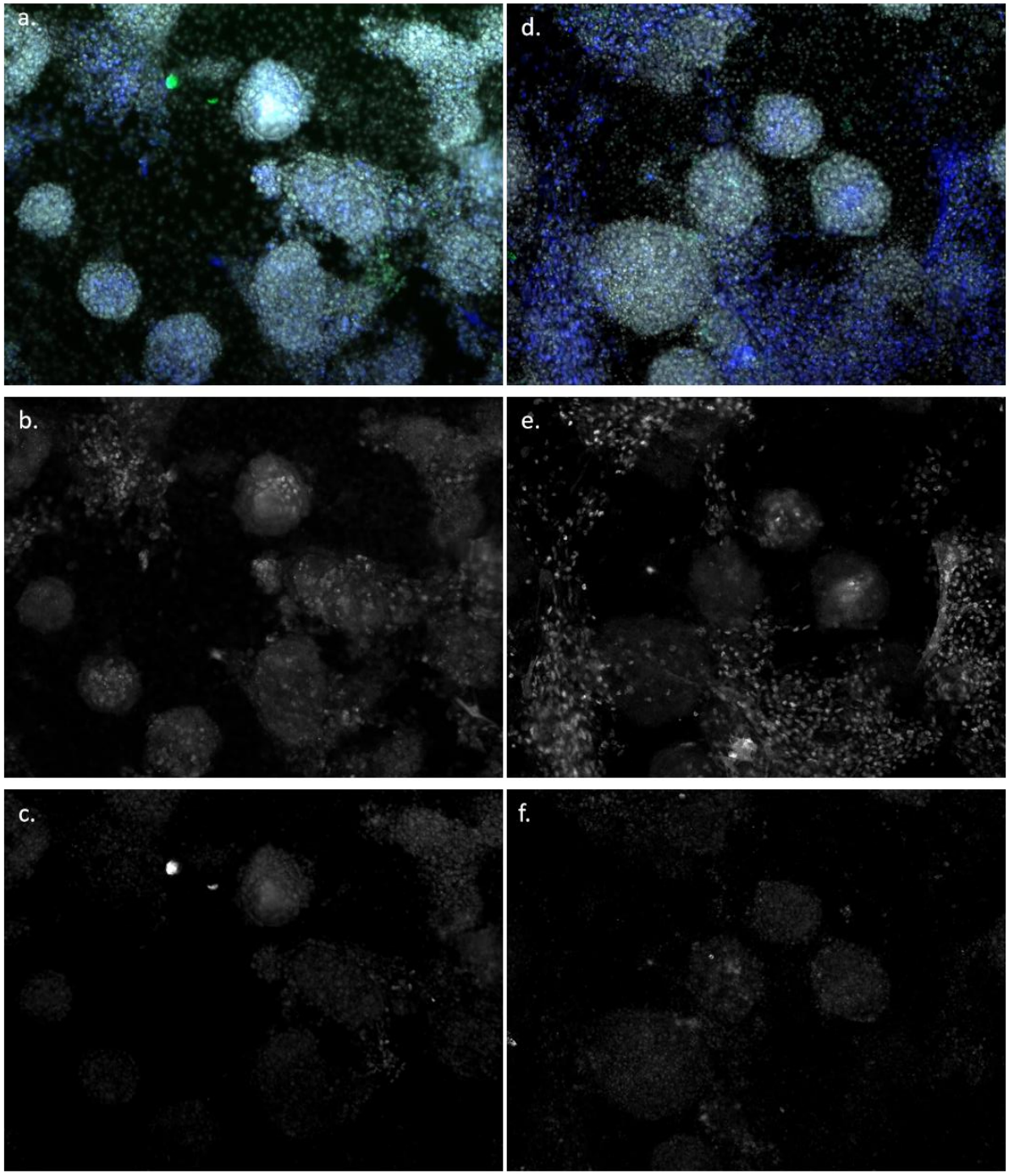


Figure.13. a&d IF analysis of mesodermal fate, with WT1 (blue) as a broad mesodermal marker and PAX2 (green) as more specified for IM, Hoechst was used to stain the nucleus of the cells (grey). **b&e.** Individual channel for WT1 expression (far-red channel). **c&f.** Individual channel for PAX2 expression (red channel). 10x magnification was used.

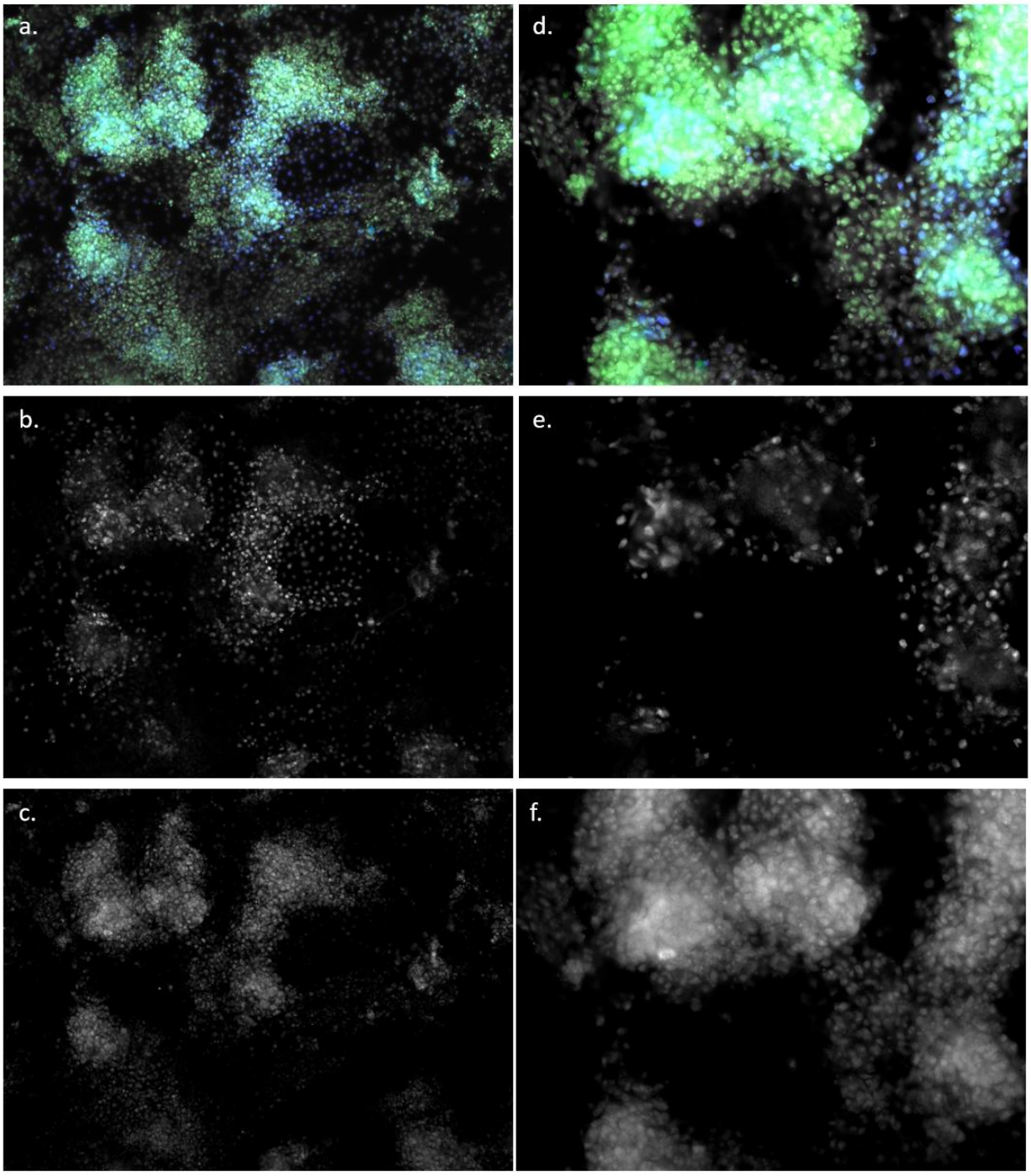


Figure.14. a&d IF analysis of mesodermal fate, with SOX17 (blue) which can be interpreted as endodermal contamination marker or cardiac progenitor cells, PAX2 (green) as more specified for IM and Hoechst was used to stain the nucleus of the cells (grey). **b&e.** Individual channel for SOX17 expression (far-red channel). **c&f.** Individual channel for PAX2 expression (red channel). **a&b&c**. 10x magnification. **d&e&f.** 20x magnification.

Early *Nr5a1* expression has been reported previously and appears to be linked to an effect of *in vitro* culturing and maintenance of pluripotency through exogenous factors (Guo & Smith, 2010). Accordingly, there was GFP signal in early EpiSCs and ESCs colonies of this line, which disappeared with the entrance to PS upon Chir treatment with (data not shown). A more careful analysis for the GFP+ cell population was conducted via FACS analysis **Figure.17.** mESCs (**Figure.17c**), AM treated cells with 100ng/ml BMP4 (**Figure.17a,d**) or non-treated (**Figure.17b,e**) were gated through the FACS machine. Surprisingly, treated and non-treated cells showed minor differences in GFP detection, but

their intensity was stronger than ESCs. After the sorting of the GFP+ cells, RT-qPCR analysis revealed that mESCs had the highest expression of *Gfp*, followed by EpiSCs and then GFP sorted cells of AM. It seems that the expression of reporter gene is not faithful, and the results do not reflect the expected output. On the other hand, when compared with mESCs, *Nr5a1* expression was 4 times higher in GFP-sorted cells, whereas EpiSC and A.M. samples had similar levels of expression, at a level half of that found in mESCs. Out of this series of experiments, several questions and problems appeared towards this cell line.

The above experiments raise several questions regarding the suitability of the *Nr5a1-Gfp* cell line. Firstly, GFP expression does not seem to follow endogenous *Nr5a1* expression, as can be clearly seen in **Figure.17f,g.** Although the GFP+ cells are expressing higher levels of *Nr5a1*, the analogy between the two expressions is missing. To further investigate this, IF analysis for NR5A1 was performed in conditions where GFP+ population of cells has been first detected (data not shown). The IF on coverslips was negative for NR5A1, but positive for other proteins (PAX2, LIM1 and SOX17). We conclude that the cell line does not faithfully report expression of *Nr5a1*, thus making it a not reliable reporter line. A possible explanation for this discrepancy can be the nature of the transgene. Indeed, *Nr5a1-Gfp* mice have been generated using a BAC construct rather than the endogenous locus and it is possible that not all enhancers required for proper transcriptional regulation are present. Although it may be normal to have RNA expression of *N5ra1* before the protein can be traced (Ikeda et al., 1994), the accumulative randomness and unreliability of the GFP signal it can lead to false clues and conclusions.

To further support this, transgenic BAC *Nr5a1-Gfp* mice from e9 to e11.5 were co-stained for GFP and NR5A1 in an IF experiment carried out by our collaborators (Neirijnck & Nef, Geneva, Switzerland). GFP was expressed in a region of the CE as early as E8.75, and thus preceded NR5A1 expression by almost one day. GFP expression continued and overlapped with NR5A1 at later time points, but GFP continued to be more broadly expressed. Only after the AP had separated from the AGP, around e10.5-11, expression of both proteins matched. This demonstrates that the cell line will be useful as a reporter for later time points when the expression of both proteins will be synchronized. We therefore concluded that for experiments of the early fate of the AGP this line is not suitable. For later screenings with more mature AP cells it may however be of use.

2.2.a.ii. *Bmp4 mutant mice.*

BMP4 has been documented to have a role in adult hormonal production (Y. Liu et al., 2017; Rege et al., 2015) and catecholamine synthesis (Otsuka, 2010). The results from human adrenal cortex are controversial, since BMP4 is not detected in array analysis of the tissue (Alarmo et al., 2013), but immunological analysis supports an expression at zG (Rege et al., 2015) and it has been suggested to have a potential involvement as a downstream target of Wnt pathway (Lerario et al., 2017). In order to further analyse the effect of the BMP pathway on adrenal development, two genetic experiments were carried out to disrupt *Bmp4* signaling. A conditional *Bmp4* floxed allele was crossed with either ubiquitously expressing inducible *CAG-CreERT* (Santagati et al., 2005) or the tissue specific *Nr5a1-Cre* line (Bingham et al., 2006). The aim of the *CAG-CreERT*, *Bmp4*$^{flx/flx}$ experiment was to temporally eliminate the Bmp4 signalling during the early steps of AGP formation and later at the AP separation and formation. This is possible through the induction of Cre activity with tamoxifen, an estrogen analogue, in targeted timepoints during the pregnancy. We decided to use the ubiquitously expressed *CAG-CreERT2* line to shut down all *Bmp4* signalling. Unfortunately, technical difficulties made the analysis of *CAG-CreERT2; Bmp4*$^{flx/flx}$ animals impossible and this approach had to be abandoned.

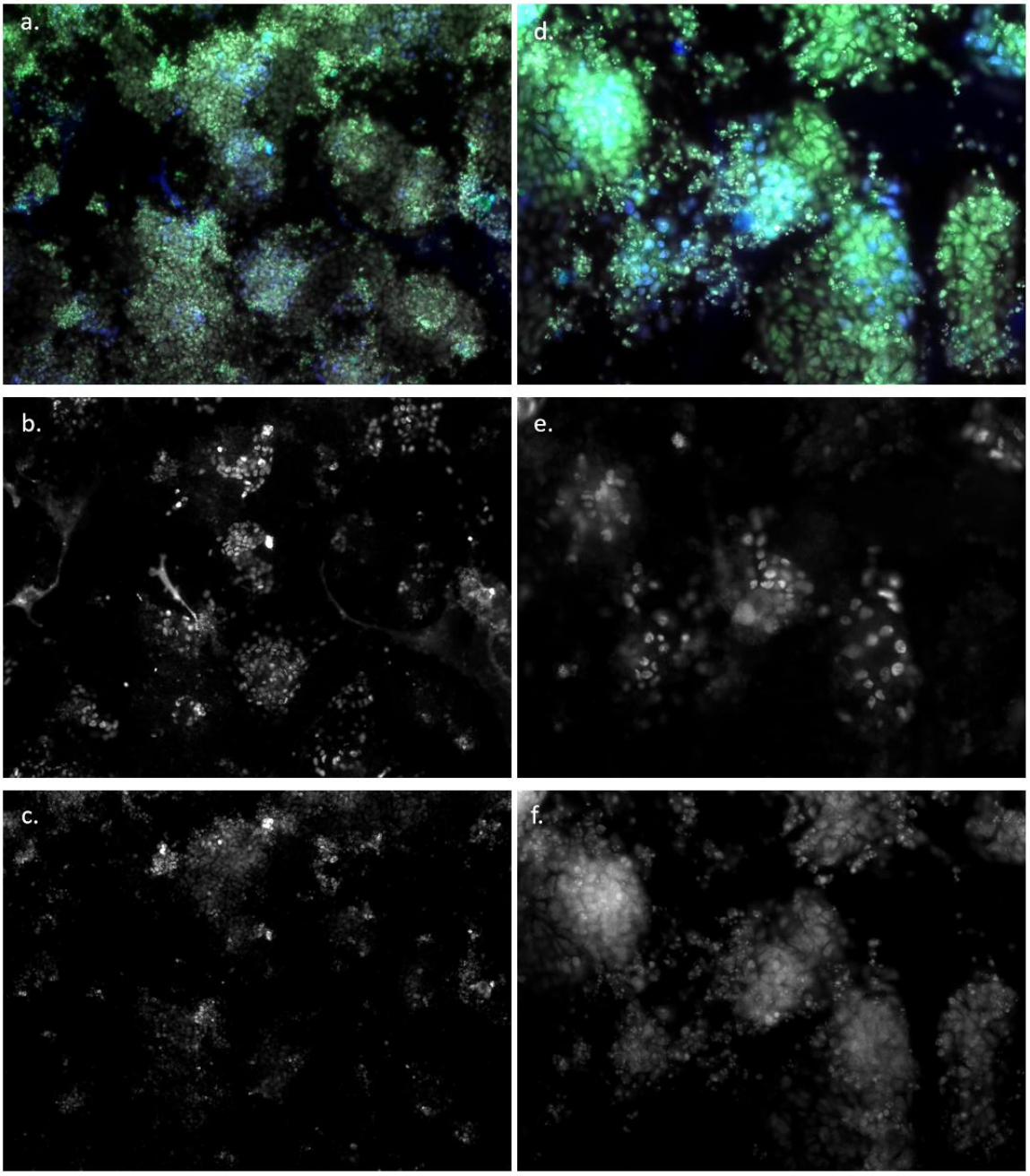


Figure.15. a&d IF analysis of mesodermal fate, with SOX17 (blue) which can be interpreted as endodermal contamination marker or cardiac progenitor cells, LIM1 (green) as more specified for IM and Hoechst was used to stain the nucleus of the cells (grey). **b&e.** Individual channel for SOX17 expression (far-red channel). **c&f.** Individual channel for LIM1 expression (red channel). **a&b&c**. 10x magnification. **d&e&f.** 20x magnification.

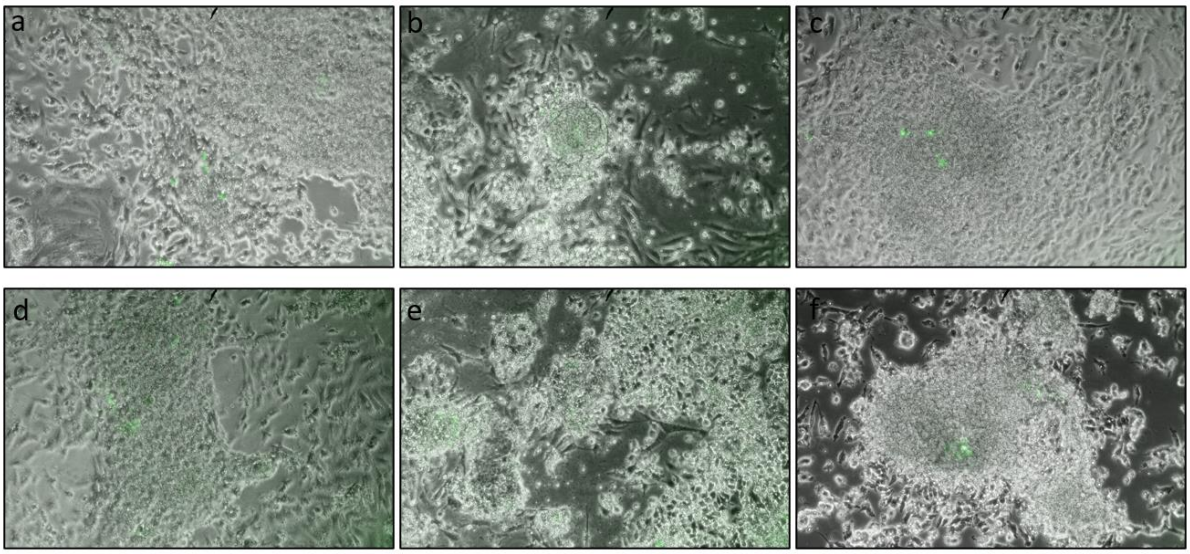
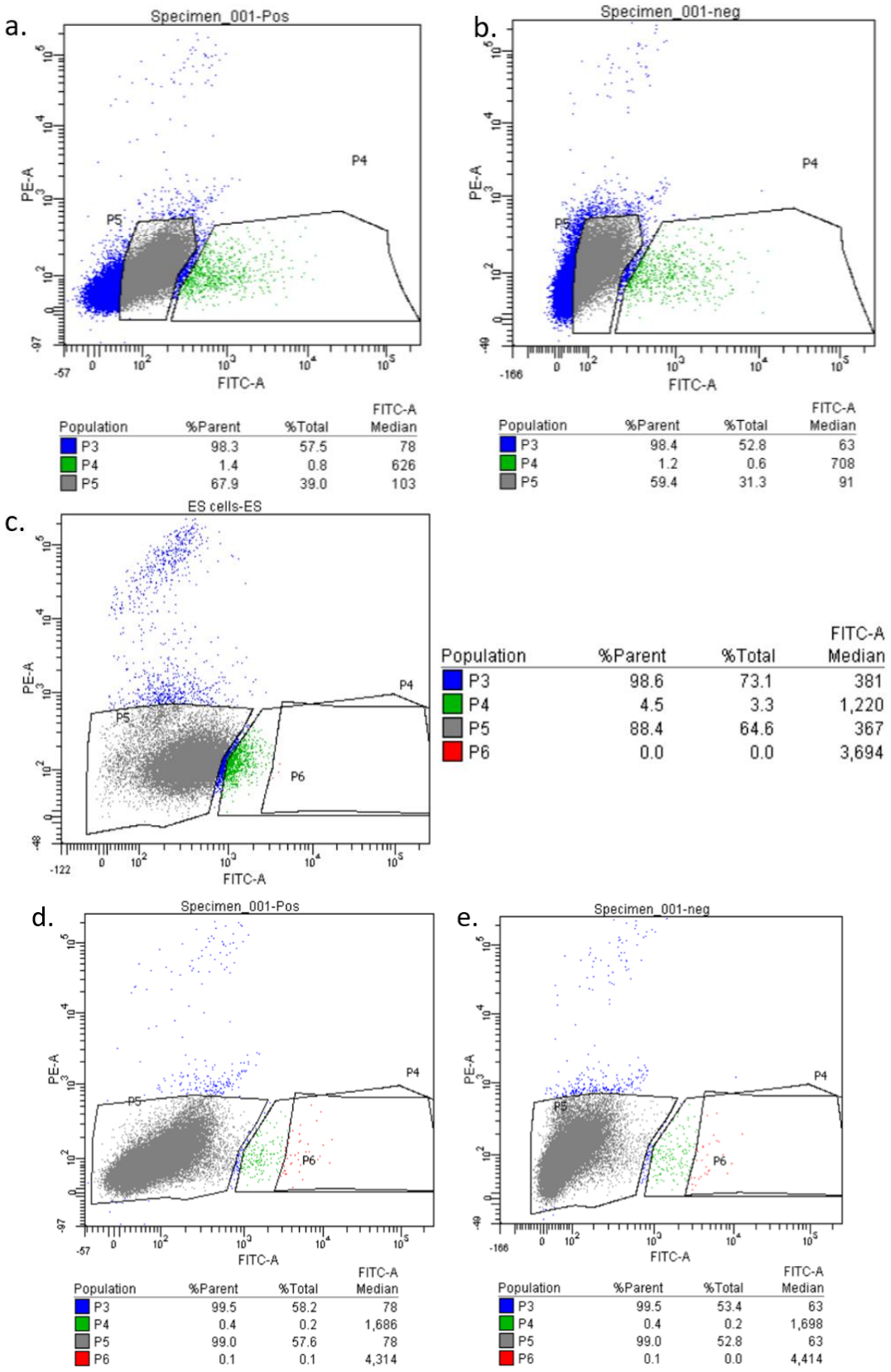


Figure.16. Live captured images of BAC-*Nr5a1-Gfp* differentiated ESCs. After reaching A.M. the cells were further treated with ascending concentration of BMP4; 1-5-10-50-100-200 ng/ml **a&f** accordingly. On green is presented the expression of the transgenic GFP.

The *Nr5a1-Cre* line was generated using the same same transgenic BAC (Stallings et al., 2002) and we expected that CRE expression in this line would follow that of the *Nr5a1-Gfp* trangsgene. Thus the CRE is expected to be present in the general area of AGP in early development, and be restricted to the steroidogenic lineage at later stages. The main aim of deleting *Bmp4* in *Nr5a1* expressing tissues was to see if endogenous *Bmp4* expression is important for the differentiation or/and maintance of the AP, at later stages of the fetal and then adult adrenal. A major concern with this crossing was that the *Nr5a1-Cre* line will follow the Gfp expression we found *in vitro* at the earliest developmental stages (ESCs and EpiSCs), which would probably result in a full knock out of *Bmp4*.

The generation of a *Nr5a1-Cre* positive *Bmp4*$^{flx/flx}$ animals yielded progeny with a normal Mendelian distribution of the heterozygous alleles. A litter size of 6-8 pups was observed, which is within the normal range for this mixed background mouse line. It is worth noting that a litter of mice was born dead and there were three Nr5a1-Cre positive Bmp4$^{flx/-}$ mice with dwarf size and visible fragibility, which died within the first two months. Additionally, the generation of the LoxP alleles in this line was not clear in the bibliography (W. Liu et al., 2004), and the indicated localisation of the LoxP sites was not faithful. As a result, the identification of floxed alleles was not possbile. Nevetherless, by crossing *Nr5a1-Cre* positive *Bmp4*$^{flx/-}$ mice 13 (8 females and 5 males) pups were raised, 7 *Cre+* and 8 *Bmp4*$^{flx/flx}$, 3 of which were Nr5a1-Cre positive Bmp4$^{flx/flx}$ (two females and one male).

Nr5a1-Cre; Bmp4$^{flx/flx}$ mice showed normal growth compared to their heterozygote and Cre negative littermates. After reaching two months of age the mice were sacrificed and analysed. No sex reversal phenotype or any overt gonadal abnormalities were observed, when compared with *Nr5a1-Cre* negative *Bmp4*$^{flx/-}$ littermates. Adrenals were of the indetical size, comperable weight (**Figure.s1**) and same morphology. Further analysis for tissue morphology using H&E staining did not reveal any abnormalities (**Figure.18**). IF staining for the steroidogenic population of the cortex and the medullary cells was performed using the markers NR5A1 and TH, respectively (**Figure.19**). NR5A1 marks the steroidogenic cells, which are expressed throughout the cortex, and TH identifies catecholamine producing cells, which is the majority of the medullar population.



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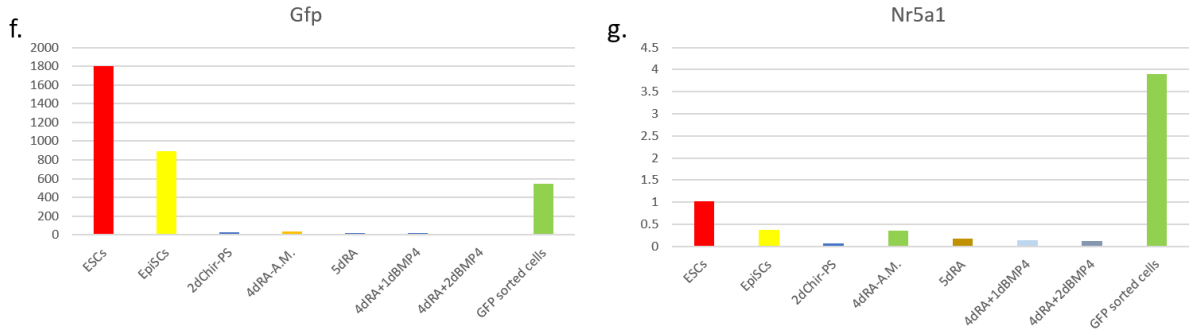

Figure.17. FACS analysis of BAC-*Nr5a1*-GFP differentiated ESCs with the additional treatment of 2 days with 100ng/ml BMP4 after reaching A.M. (**a**), and non-treated (**b**) for comparison. ESCs of this line were also FACS analysed in order to remove the background GFP expression of the line (**c**). Further analysis with the new parameters was done with treated (**d**) and non-treated cells (**e**). Comparative expression of Gfp was done between GFP sorted and samples from previous steps of differentiation (**f**); Nr5a1 expression was analysed also (**g**) in comparison with the initial expression from the ESCs and onwards.

The patterned expression of these two proteins does not show any disruption of the two tissues or any developmental defect. On both genders, NR5A1 nuclear staining was mostly found on the adrenocortical cells, with some cells in the medullary region having a cytoplasmic one (probably artefact). On **Figure.19a** there seems to be a partial mix of the cortical and medullary region or a separation of the medulla by a cortical layer of cells, but apparently this can be either the plain of section or a normal event on some animals. TH was predominantly on the medullar region, but in very rare cases like **Figure.19c** TH+ cells can be found close to the borders of zG and zF. Apart from that, there was nothing abnormal with its expression in either sample of both genders.

The final analysis of the Nr5a1-Cre positive Bmp4$^{flx/flx}$ adrenals was on their zonation. IF for zG (DAB2) and zF (CYP11B1) specific markers was conducted to investigate wether the zonation was disrupted or abolished or if they exhibited a differntiation deffect (**Figure.20**). As expected, CYP11B1 was strongly localised to the zF and with a weaker staining in zG. On the other hand, DAB2 specifaclly stained zG cells and some medullary localised cells, while some possibly migrating cells were osberved at zF. Once again we did not observe any major difference between the mutants and the controls.

The lack of an obvious phenotype suggests that endogenous expression of *Bmp4* within *Nr5a1* expressing cells during AGP developemnt and later on within the adrenal gland is dispensable. It has been speculated that because BMP4 is expressed at the adrenal cortex, it may affect the homeostasis of the tissue by autocrine fucntion (Rege et al., 2015). In this experiment we aimed to abolish any *Bmp4* expression from steroidogenic tissue, and we did not see this affecting the development and the homeostasis of the tissue on adult mice. Since we did not investigate the earlier developmental stages, we cannot conclude if there is an earlier defect which later gets compensated. It should be stressed, that our analysis was strictly, histological and a thorough investigation of RNA and hormone levels and the steroid production may reveal more subtle differences. In addition, only 3 mutant mice were analysed and additional animals are needed to confirm this observation. Finally, our inability to faithfully recognise the depleted Bmp4 gene on the mutants through PCR can greatly impact our conclusions.

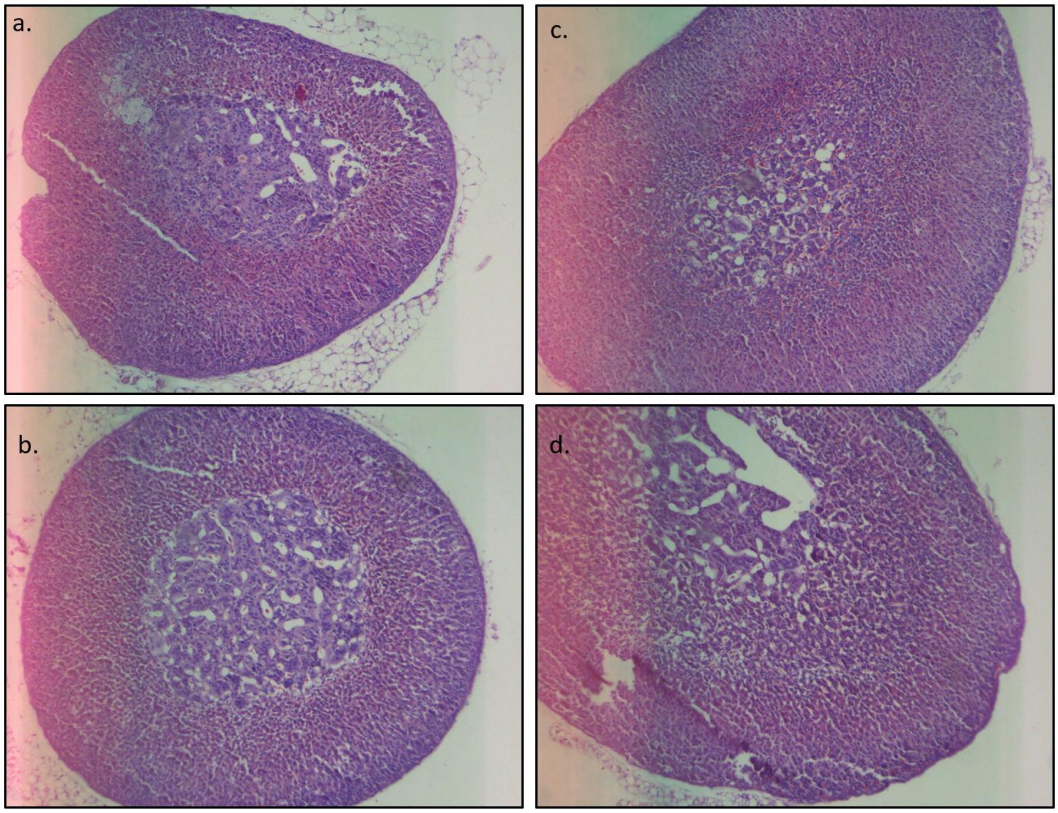

Figure.18 Haematoxylin and eosin (H&E) staining of adrenals from 2 months old mice; **a&b** males, **c&d** females. **a&c** Nr5a1-Cre *negative* Bmp4$^{flx/+}$, **b&d** Nr5a1-Cre *positive* Bmp4$^{flx/flx}$.

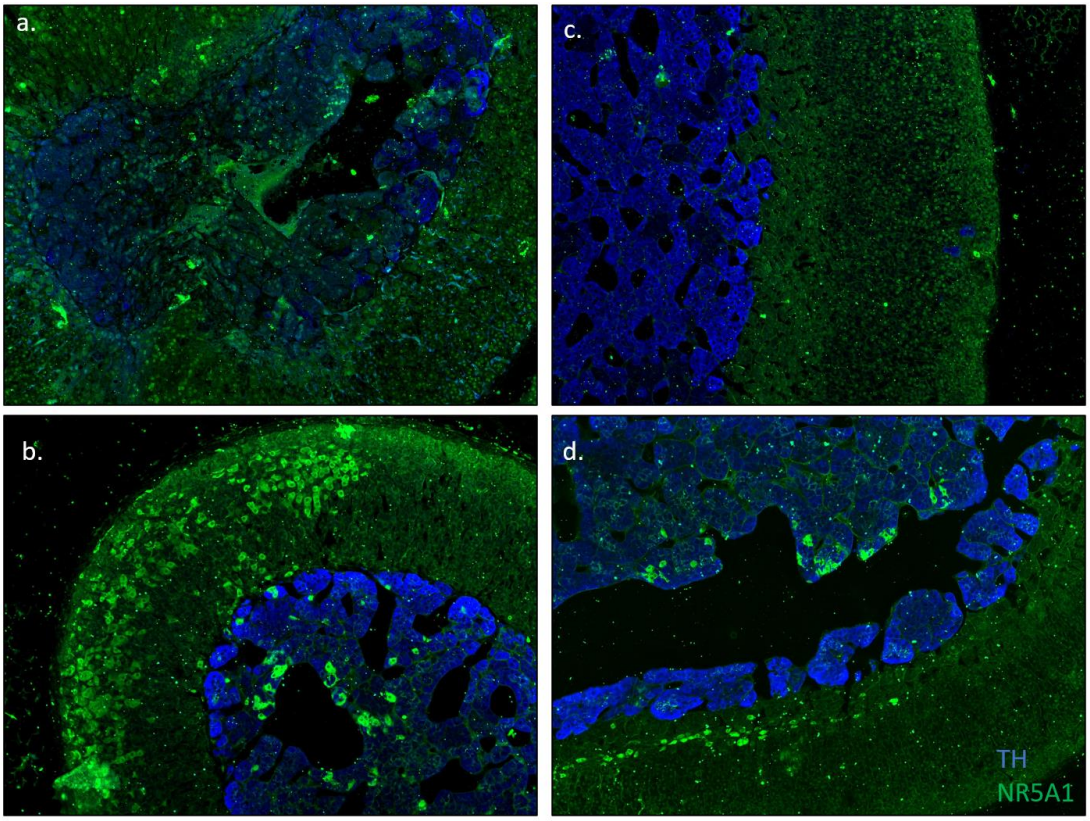

Figure.19. IF staining for TH (Tyrosine Hydroxylase), marker of the adrenal medulla, and NR5A1 of adrenals from 2 months old mice; **a&b** males, **c&d** females. **a&c** *Nr5a1-Cre negative Bmp4$^{flx/+}$*, **b&d** *Nr5a1-Cre positive Bmp4$^{flx/flx}$*.

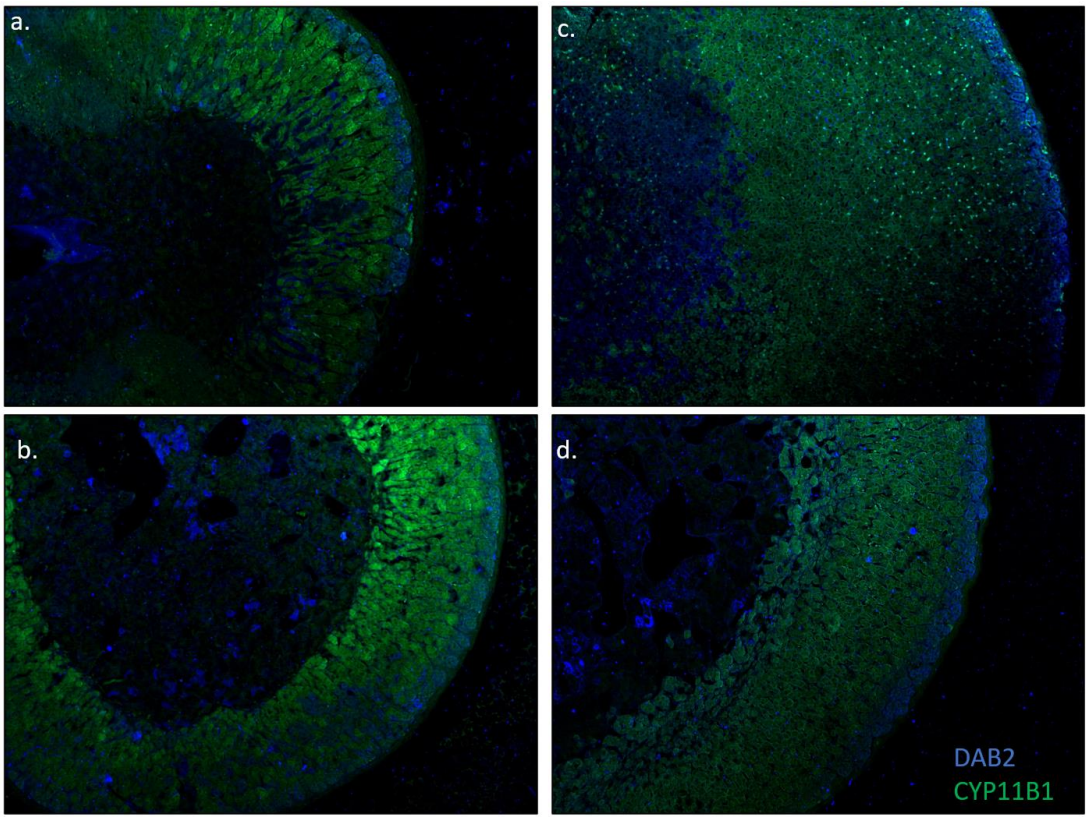

Figure.20. IF staining for DAB2 (zG marker) and CYP11B1 (zF marker) of adrenals from 2 months old mice; **a&b** males, **c&d** females. **a&c** *Nr5a1-Cre* negative *Bmp4*$^{flx/+}$, **b&d** *Nr5a1-Cre positive Bmp4*$^{flx/flx}$.

Apart from the BMP pathway, other major differentiation pathways were tested in our *in vitro* differentiation system. Various recombinant proteins from the BMP, WNT and FGF signalling molecules, and chemical inhibitors and activators of these pathways were introduced in different combinations and concentrations in A.M. staged *in vitro* cultures (**Figure.s2)**. Furthermore, A.M staged cells were allowed to interact with each other in aggregates and cultured in 3D on Transwell filters as described in **Figure.23**. Pathway activators and inhibitors were also tested under these conditions. In all those trials ARPMI was used as the base medium. Unfortunately, no significant upregulation of *Nr5a1* could be detected by RT-qPCR analysis or at the protein level (IF staining). *Mc2r* RNA, however, showed responsiveness to the above-mentioned treatment. A main conclusion from these experiments is that induction of the WNT pathway (treatment with Chir 3um) at the A.M. stage leads to the downregulation of potential AGP markers. An example of this effect can be seen in **Figure.24a,b,** where cells in medium containing RA + FGF2 were compared with and without Chir treatment.

2.2.b. Creation of Mc2r reporter cell line.

As mentioned above, during our trials for the effect of BMP pathway on AGP formation, we realised problematic nature of the *Nr5a1-Gfp* mESCs line for screening purposes, thus a new reporter line was needed. During the *in vitro* differentiation experiments, we recorded the early expression of *Mc2r* (**Figure.11b**, which is an interesting observation considering the tissue specificity of this gene at later stages of development. MC2R forms an active complex with its accessory protein (MRAP) and acts as a receptor for ACTH signalling, to modulate the downstream production of cAMP. *Mc2r* has a specific expression on fetal adrenal at e14.5. At later stages and in the adult adrenal expression becomes

restricted to the zF. Its expression has been linked with the normal adrenal gland development and homeostasis, where in its absence the progeny has defects resembling *Nr5a1* mutants (Chida et al., 2007). Older studies have indicated a much earlier expression of *Mc2r* in the UR area and lungs at e11.5 of mouse (Nimura et al., 2006). Although the spatial expression of *Mc2r* in their results of e11.5 is broader, the fact that they also detect it, indicates that it may not be an artefact of the *in vitro* system. At the same time, single sequencing data from our collaborators (courtesy of S. Nef), demonstrated *Mc2r* expression within the UR from e9 onwards, with more restricted expression to steroidogenic tissues at e10.5. Therefore, RNAscope, an *in situ* hybridization technique, for *Mc2r* was performed for E9.5, e10.5 and e14.5 mouse embryos. Only at e14.5 (**Figure.s3)** there was a specific staining, while at E9.5 and e10.5 there was nothing noticeable (data not shown).

We next set out to examine the spatial and quantitative expression of *Mc2r* on a E9.5 pool of mouse embryos. The embryos were dissected manually into different compartments (**Figure.21c**), RNA extracted under sterile conditions and analysed via RT-qPCR, which were then compared with *in vitro* samples of previously tested differentiation protocols and e12.5 adrenal glands (**Figure.21a,b**). It is apparent that *Mc2r* is more expressed in the caudal, and more specifically in the specified UR of the developing embryo. *In vitro* differentiated samples showed an even greater enrichment of 20-fold in 2D and around 70-fold after 3D culture, which is comparable to later stages of adrenal development (**Figure.21a**). Interestingly, *Nr5a1* follows *Mc2r* expression pattern *in vivo* (adrenal sample was removed, because of 10^3 value), but not *in vitro.* Indeed, cells cultured in 3D appeared to have lost all *Nr5a1* expression and 2D samples showed expression lower than that found in s.UR. Furthermore, when combined with results from enriched mesodermal culture *in vitro,* these results showed that *Mc2r* is unlikely to mark cells outside of the UR. It will, however, be interesting to understand why *Nr5a1* does not follow *Mc2r* expression *in vitro*, and if by following and studying its expression we can further understand the early development of the AGP.

Given the promising pattern and specificity of *Mc2r* expression and its important role for adrenal function *in vivo*, it appears an ideal candidate for a reporter gene. Since heterozygotes for *Mc2r* seem to not have any serious defects on adrenal (Chida et al., 2007), we aimed to knock-in a *Gfp* gene on its recorded common promoter/regulatory region (Chida et al., 2007; Shimizu et al., 1997)(**Figure.s4)**. To increase recombination efficiency, a CRISPR/Cas9 approach was used to insert the transgene in Bl6 mESCs. A *Mc2r-Gfp*/+ Bl6 mESC line was created and validated with PCR and Sanger sequencing for proper insertion. Functional analysis of the close was thereafter performed. The clone at ESC stage showed normal growth, but induction of differentiation to A.M. using conditions established for the R1 mESC line showed a high amount of cell death. RT-qPCR analysis revealed a delayed and reduced *Mc2r* expression compared to Bl6 and R1 cells, probably because of the heterozygote knock-out that was created by the knock-in (**Figure.22g**). Also, the expression of *Nr5a1* seems abnormal with a first time seen peak at the PS stage (**Figure.22h**). This abnormal behaviour was also confirmed by a later FACS analysis for A.M. staged *Mc2r-Gfp* cells, where there was no detectable GFP signal (**Figure.22b**). Because the *Mc2r-Gfp* cloned did not differentiate properly, an alternative method to assess the insertion and functionality of GFP was needed. As described above, *Mc2r* RNA is present significantly before its protein in steroidogenic lineage cells, and we can assume that the GFP protein may follow the MC2R expression. As already mentioned, direct differentiation into steroidogenic cells has been achieved in several studies by overexpressing NR5A1 using a transgene (Crawford et al., 1997; Ruiz-Babot et al., 2018; Sonoyama et al., 2012; Yazawa, 2014). Therefore, we aimed to directly differentiate

the mESCs to steroidogenic ones and test for GFP expression. mESCs were plated in feeder-free condition and transfected with an *Nr5a1* expression plasmid (courtesy of E. Lalli, Sophia Antipolis, France), and cultured in GMEM medium containing 8-Br-cAMP and RA. Bl6 cells were used as negative control and the BAC-*Nr5a1-Gfp* line, transfected with the *Nr5a1* expression plasmid, as positive control. Bl6 cells were also transfected with a GFP overexpressing plasmid to act as a positive control for the transfection. Meanwhile, *Mc2r-Gfp* cells were also subjected to our *in vitro* differentiation protocol and used in this analysis for comparison.

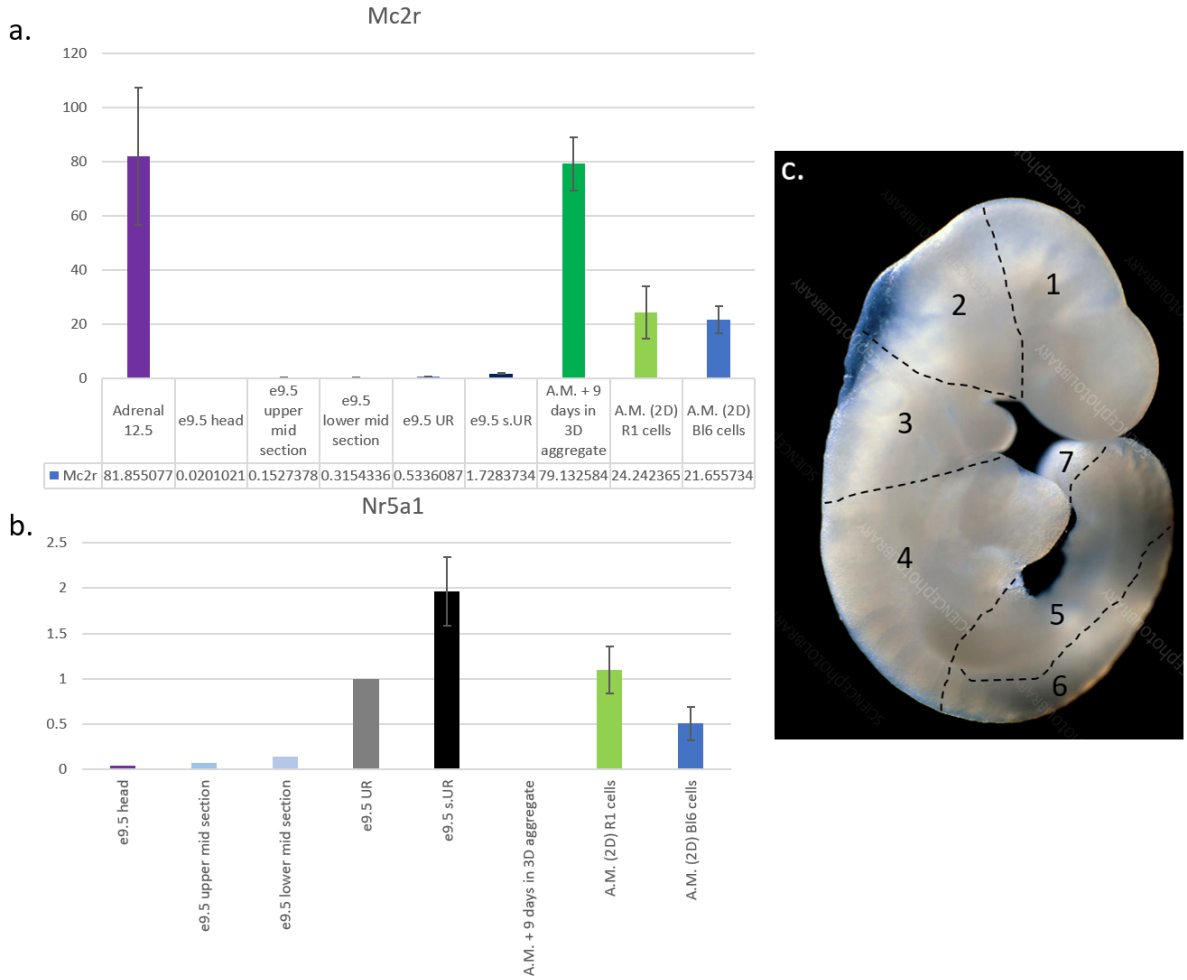

Figure.21. RT-qPCR analysis of *Mc2r* (**a**) and *Nr5a1* (**b**). Different regions of a E9.5 mouse has been used in ordered to spot the specificity of their expression (**c**). **c**. Part 2 represents the head, part 3 the upper mid section, part 4 the lower mid section, part 5 the s.UR and together with part 6 the UR. The qPCR results are compared with samples of the entire E9.5 mouse (a) and the UR (b) and normalised against *Sdha* expression.

The day after transfection, both positive controls and the *Mc2r-Gfp* clone had detectable and specific GFP expression, as examined under a fluorescent microscope, which was not the case for cells differentiated according to the A.M. protocol (data not shown). Two days after transfection all samples were subjected to FACS analysis. Bl6 (**Figure.22a**) and *in vitro* differentiated *Mc2r-Gfp* cells (**Figure.22b**) did not reveal detectable levels of GFP; while GFP transfected cells (**Figure.22c**) had a strong signal showing a ~25% transfection efficiency. Both BAC-*Nr5a1-Gfp* (**Figure.22d**) and *Mc2r-Gfp*

(**Figure.22e**) clones that had been transfected with the *Nr5a1* expression plasmid showed significant GFP expression (15% and 12% of their population, respectively).

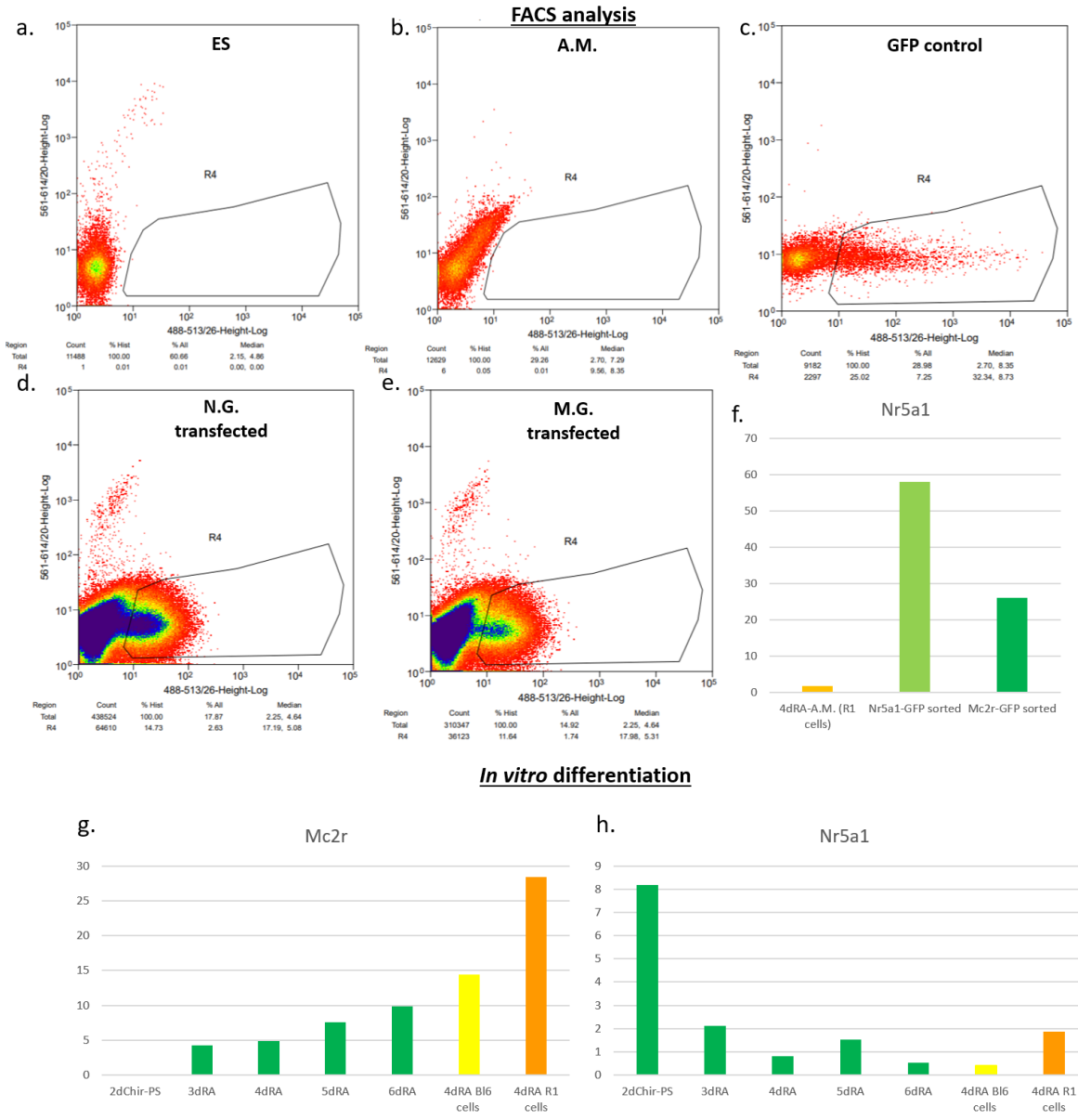

Figure.22. FACS analysis of *Mc2r*-GFP generated cell line. **a.** ESCs. **b**. A.M. differentiated cells. **c.** transiently expressing GFP cells via transfection. **d**. *BAC-Nr5a1-GFP* induced differentiated cells with *Nr5a1* overexpression. **e.** *Mc2r*-GFP induced differentiated cells with *Nr5a1* overexpression. Sorted cells from **e**&**d** underwent RT-qPCR analysis for *Nr5a1* (**f**) and *Mc2r* expression (data not shown, no expression). Analysis of the *Mc2r-Gfp* clone for the *in vitro* differentiation protocol in different timepoints during the A.M. induction, in comparison with Bl6 and R1 cells for *Mc2r* (**g**) and *Nr5a1* (**h**). All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised to *Sdha* expression.

Extrapolating from the transfection control (25% transfection efficiency), approximately half of the successfully transfected cells expressed GFP. This result demonstrate that the inserted *Gfp* construct is functional and can be activated upon differentiation into steroidogenic cells. GFP positive cells were sorted and further analysed for the relative expression of *Mc2r* and *Nr5a1* using RT-qPCR (**Figure.22f**). Surprisingly, none of the sorted samples exhibited *Mc2r* expression (data not shown), and the observed Nr5a1 expression may reflect residual expression from the transfected *Nr5a1* plasmid. Possible explanations for the GFP detection and absence of *Mc2r* can be either that the steroidogenic

profile has already shut down (detection of residual GFP protein), or that *Mc2r-Gfp* expression is activated before the expression of *Mc2r*. We should also keep in mind that this is not an optimized protocol, but just a quick way to imitate the steroidogenic profile of a cell. Cells generated this way may lack functionality and more specifically, MC2R expression (RNA and protein level) and activity. The genetic validation of the proper insertion of GFP construct and the expression of GFP after *Nr5a1* transfection makes this line a potential tool, which however will need further optimization.

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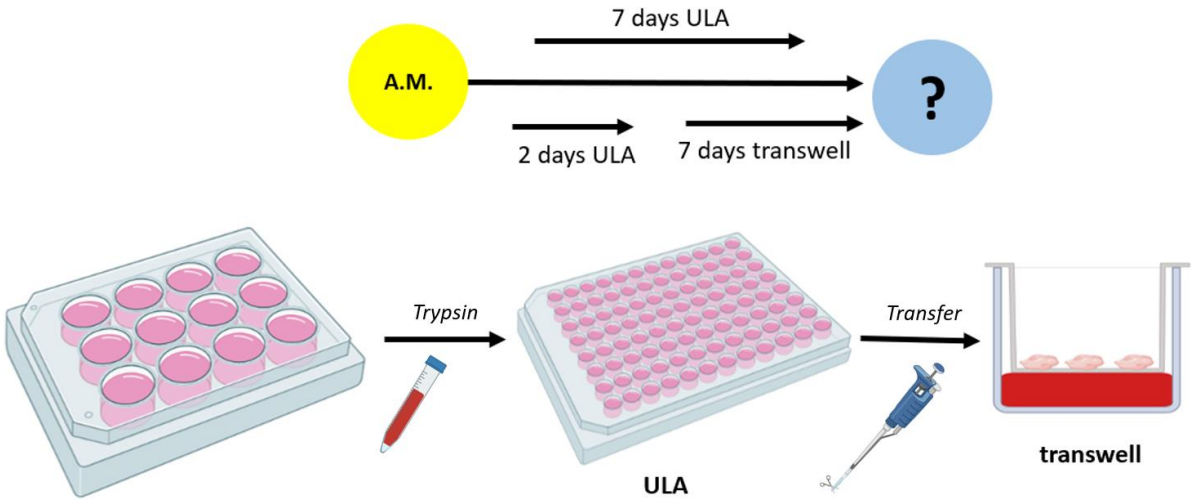


Figure.23. Illustration of aggregate formation from 2D A.M. state (4dRA). Cells were trypsinized with 50% trypsin and moved to ultra-low attachment (ULA) 96 well plates at concentration 8*10^4 cells/well. From that point the aggregates were either continued treated in the ULA plates or they were moved to transwell plates (6/well) by pipetting them up and gently placing them on the filter. ARPMI was used as medium the whole procedures.

2.2.c. SHH treatments of A.M.

Thereafter, for all further *in vitro* experiments R1 cells were used unless indicated otherwise. All previous pathways (activators and inhibitors) were also tested on the R1 cell line and gave similar results. We next turned our attention to the Hedgehog (hh) pathway, as SHH is known to be required for the recruitment of steroidogenic cells during development (Guasti et al., 2011; King et al., 2009; M. A. Wood et al., 2013a). Both, 2D (**Figure.11a**) and 3D (**Figure.23**) systems were tested. Firstly, we wanted to see if cells are responsive to SHH by measuring the expression of the steroidogenic markers *Nr5a1* and *Mc2r* (**Figure.24a,b**). Cells of A.M. stage were treated with the indicated factors in ARPMI medium for two days and later analysed by RT-qPCR. From the results we can see that cells are responding to SHH and can maintain Nr5a1 and Mc2r expression, while if they are kept in the same culture condition as A.M. *Mc2r* increased, but *Nr5a1* expression was lost. Furthermore, low concentrations of Chir seem to totally inhibit the steroidogenic fate of the cells, which is in agreement with our previous data (page 51).

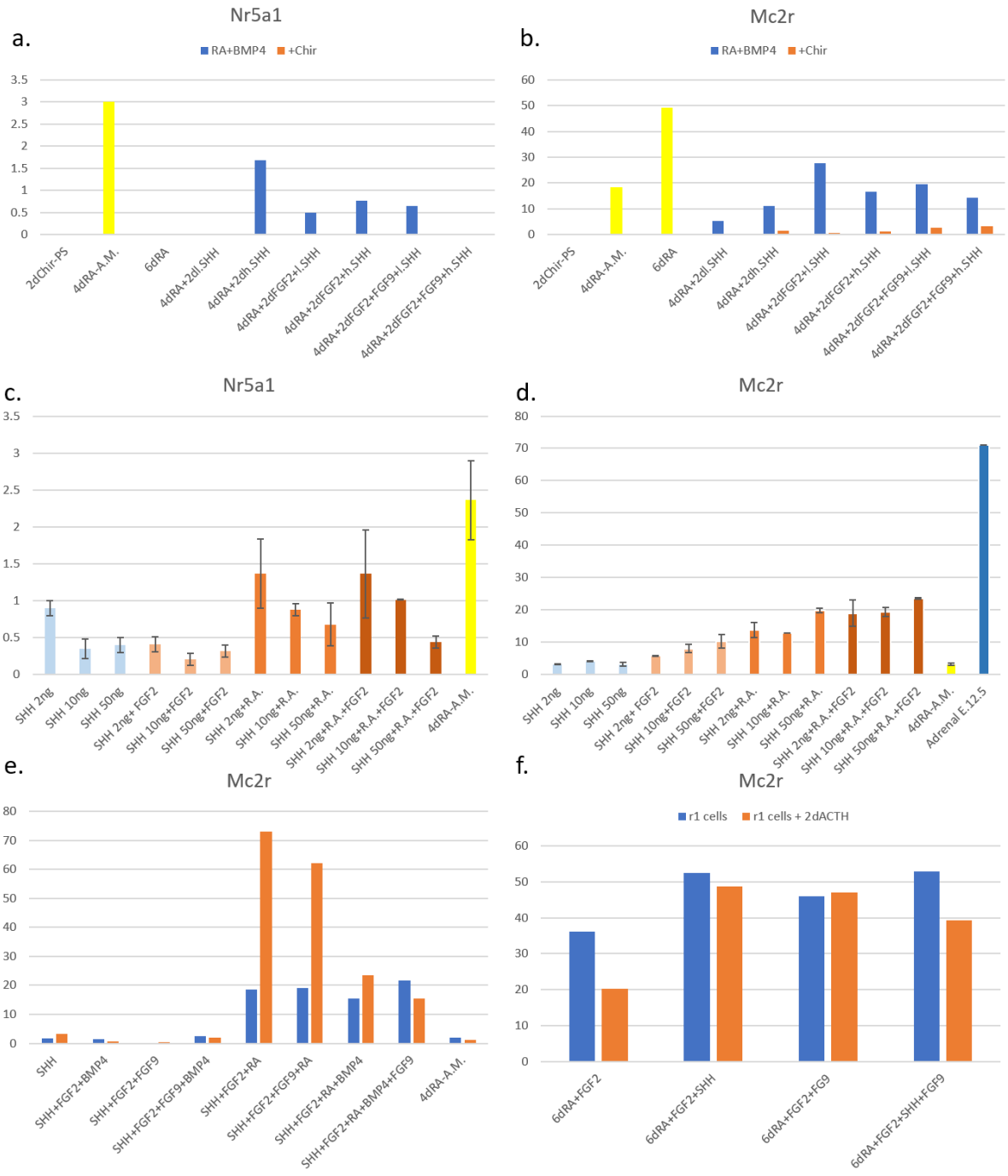


Figure.24. **a&d** treatments on 2D cell culture, **e&f** 3D formation and treatments. **a&b**. Cells were kept on RA (1uM) +BMP4 (100ng/ml) medium and were compared with the Wnt pathway inductor (Chir 3uM), on different conditions and combinations of SHH (l=10ng/ml, h=50ng/ml), FGF2 (100ng/ml) and FGF9 (100ng/ml), for 2 days. After, *Nr5a1* (**a**) and *Mc2r* (**b**) expression were measured. (**c&d**) Supplementary experiment on different conditions of SHH. **c.** *Nr5a1* and **d**. *Mc2r* relative expression is shown. **e&f**. Cells were formed in spheroids and then moved to transwells. **e**. Spheroids were treated for further 6 days with different combinations of SHH (10ng/ml), FGF2 (100ng/ml), FGF9 (100ng/ml) and RA (1uM); relative expression of *Mc2r* is shown from two experiments. **f**. Conditions of (**e**) with highest *Mc2r* expression were tested for response in ACTH signalling; relative expression of *Mc2r* is shown All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised to *Sdha* expression.

Since this was the first time we observed maintenance of *Nr5a1* and *Mc2r* expression after the A.M stage, SHH seemed to merit further investigation. With the same experimental setup, different concentrations of SHH with combinations of RA and FGF2 were tested; RA and FGF2 were chosen since

in this condition there is already expression of *Nr5a1* and *Mc2r*, which was lost during treatments. The samples were then gathered after two days of treatment and their relative expression of *Nr5a1* (**Figure.24c)** and *Mc2r* (**Figure.24d)** was tested via RT-qPCR. It is obvious in the presence of RA both genes have the highest expression levels when compared to the rest of the conditions. Interestingly, *Nr5a1* had the highest expression on the lowest SHH concentration, whereas the reverse was the case for *Mc2r*. Based on these data, we chose the 10ng/ml SHH concentration for 3D experiments. Aggregates were formed in ultra-low attachment plates and moved onto transwell filters, as outlined in **Figure.23**. Medium containing the corresponding factors were changed every two days (**Figure.24.e,f**). RNA was then isolated and subjected to RT-qPCR analysis. Surprisingly, *Nr5a1* expression was completely lost in all conditions, whereas *Mc2r* expression significantly increased in the presence of RA. MC2R was however unlikely to be functional, as its co-receptor *Mrap* was not found to be expressed nor was *Nr5a1* upregulated when treated with ACTH. Taken together, SHH in combination with RA was the combination found to have a positive effect in this *in vitro* differentiation system. Although there was not a significant steroidogenic fating of the cells based on their profile, one can speculate that this condition can be a bridge for future treatments. The fact that *Nr5a1* expression is lost upon prolonged treatment or in 3D cultures shows that additional factors are required to trigger proper differentiation of A.M. cells into AGP-like and/or steroidogenic cells.

2.3. ECM on steroidogenic fating.

2.3.a. Screening for suitable ECM.

Until this point our research was focused on tinkering with signalling pathways known to be present in the E9-9.5 embryo in the region of the future AGP. While promising, our results so far were insufficient for proper differentiation NR5A1 producing cells. We next decided to add an extra dimension to our *in vitro* protocol, by including the ECM as a parameter. Our standard differentiation protocol up to A.M. is carried out on Geltrex ECM, which is mainly composed of Laminin, Collagen IV, Entactin and Heparan sulphate proteoglycans. To introduce the new matrix to the cells, A.M. staged cultures were trypsinized, pooled together and then replated in proper concentrations after counting (**Figure.25**). Commercially available ECM screening plates bearing 96 different ECM conditions were used as the initial tool to investigate potential ECM candidates (**Figure.25a**). Each condition of this plate represents a different combination of glycosaminoglycan and specific peptide of an ECM protein, which has been shown to be of importance for the ECM identity and characteristics. The plating concentration of $8*10^4$ cells per well of 96 well plate was established as optimal for this protocol. Visual examination revealed that changing the ECM stimulates the A.M. to different fates by examining their phenotypical appearance (**Figure.27**). As a general rule, cells on Dextran and Heparin were surviving mostly as colonies, which was not the case for Dermatan and Chondroitin. Furthermore, there was a pattern on the final population of cells, which can reflect survivability and proliferation, according to the peptide the cells were cultured on. A good example was the Collagen and FGF peptides, which appeared to increase proliferation and thus resulted in overpopulation. Also, under specific conditions the cells were more easily detaching from the matrix, even during the changing of the medium. Unfortunately, the information provided by the company manufacturing the plates is limited and we could not know what those peptides are.

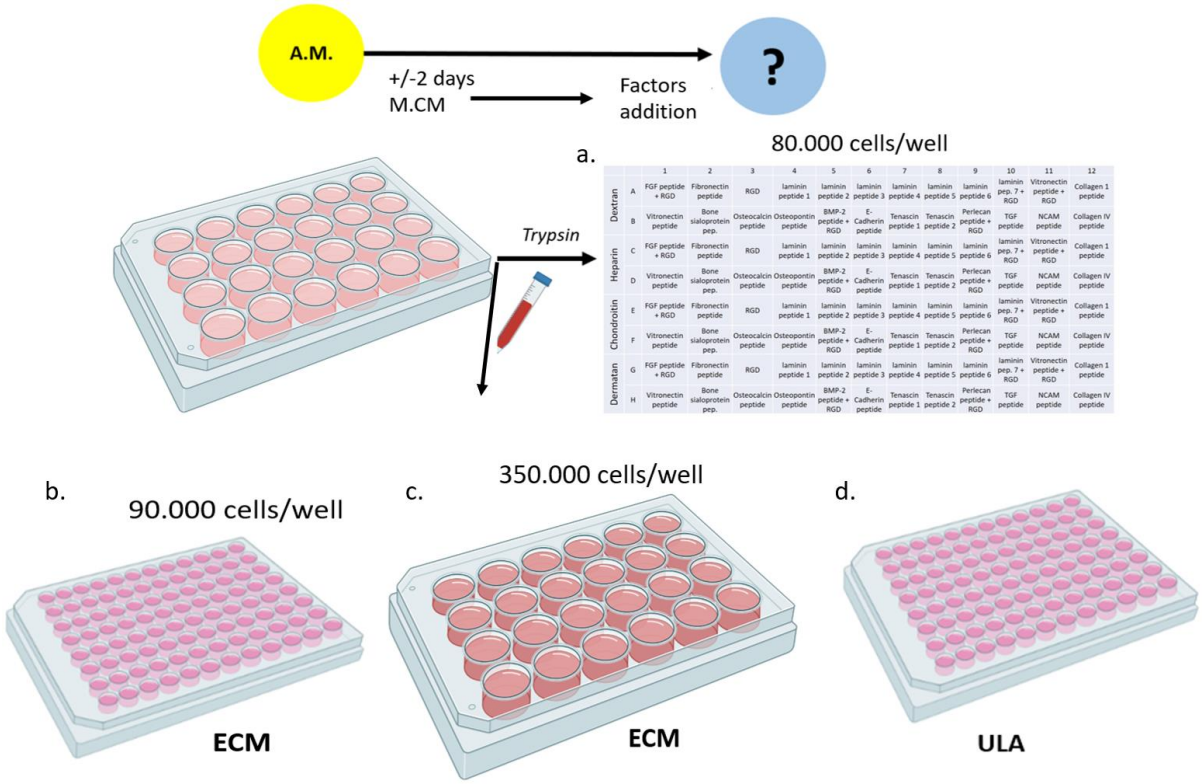


Figure.25. Cells of A.M. stage were trypsinized with 50% trypsin and plated in different conditions, and either retained 25% of their medium or not. **a**. Cells were plated on a multi-ECM 96 well plate. **b-c**. Cells were plated on custom coated 96 or 24 well plates. **d**. Cells formed aggregates in ULA plates.

IF was chosen as the screening method since the main aim of this protocol was to have expression of NR5A1 and thus AGP cells. As a first test, we performed IF analysis on adrenocortical cells (**Figure.26**). The staining reveals a strong nuclear and perinuclear signal of NR5A1. Remarkably, the screening revealed NR5A1 positive cells in different conditions. In some conditions nicely formed colonies expressing NR5A1 could be detected (**Figure.28-29**). Co-staining with GATA4, a general marker of CE and AGP at that stage, revealed cells that were positive for both markers (**Figure.28**). The effect of ECM on the cells is visible on the IF staining, since there is a variability on the number of colonies, their size, and the existence of cells outside of a colony. Furthermore, only a proportion of cells were co-stained for NR5A1 and GATA4, as can be clearly seen in **Figure.28-29a**. This may indicate that a colony may consist of on the one hand AGP-like cells (co-expression of NR5A1 and GATA4) and on the other hand a more advanced differentiation state, when only NR5A1 can be detected. The fact that a large proportion of NR5A1-negative colonies exhibit GATA4 expression may indicate that also these cells have the potential to develop into NR5A1 producing ones given the correct stimulus. In some conditions no NR5A1 positive cells could be detected, but even for positive wells the results were not highly reproducible. Combined with the fact that we cannot know what those peptides are, we decide to switch to ECMs which prepared in our lab, based on the information already gathered from the commercially available ones.

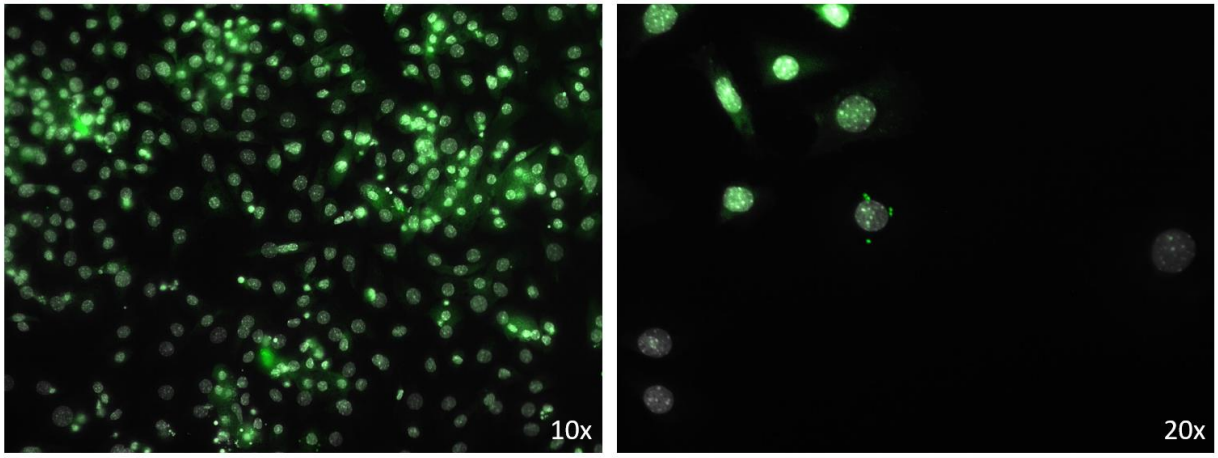


Figure.26. IF staining of NRA1 for isolated adrenocortical cells. At 10x magnification there is a clearly nuclear staining, and with 20x magnification stronger spotted nuclear and perinuclear staining can be observed.

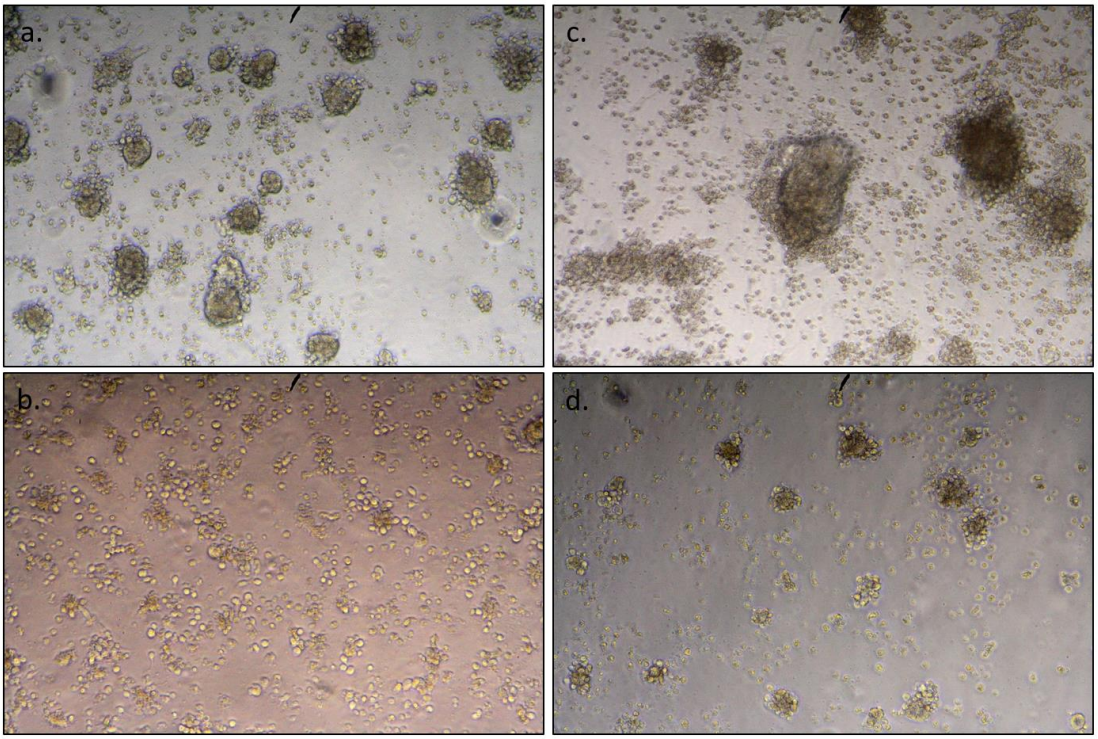


Figure.27. Brightfield images from cells plated in **figure 25.a. a.** Tenascin peptide-2 + Dextran. **b.** Laminin peptide-4 + Dermatan. **c**. Laminin peptide-5 + Heparin. **d**. Collagen peptide-1 + Dermatan.

Therefore, cells were plated as in **Figure.25b** on defined ECMs. Tenascin-C (TNC), Fibronectin (FN1), Collagen-1 (COL1), Vitronectin (VTN) and the commercially ready ECMs of gelatine, Geltrex and Matrigel were used in different combinations with each other or alone, and with or without the presence of Heparin as supporting glycosaminoglycan. As screening we again used the expression of NR5A1 as detected by immunofluorescent analysis. The commercially available ECMs of gelatine, Geltrex and Matrigel failed to show any NR5A1 expression at any the studied conditions, while COL1 had positive result only when combined with TNC or FN1. NR5A1 positive cells with the addition of Heparin were located on VTN and FN1/FN1+COL1 conditions Finally, only VTN and FN1 could give rise to NR5A1 expressing cells. The majority of the NR5A1 positive cells in any condition were found to grow within colonies.

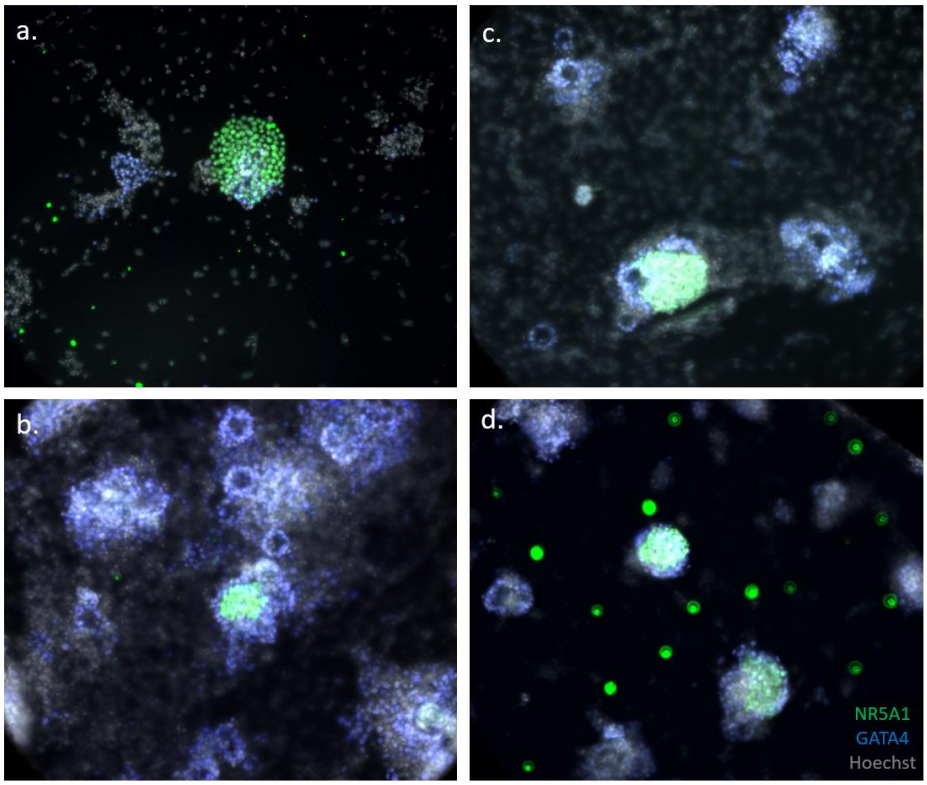


Figure.28. IF staining for NR5A1, GATA4 and Hoechst from cells plated in **figure 21.a;** cells were analysed after 7 days on the ECM plates with ARPMI medium. **a**. Tenascin peptide-2 + Dextran. **b**. Fibronectin peptide + Heparin. **c**. RGD + Dextran. **d**. Osteopontin peptide + Dermatan.

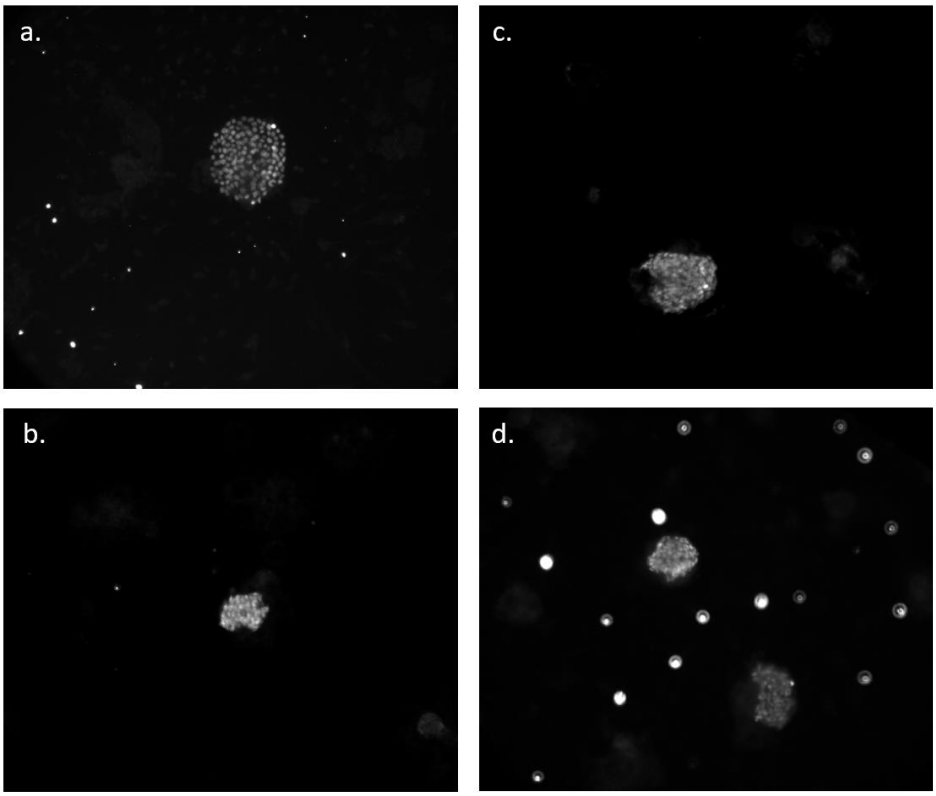


Figure.29. IF staining for NR5A1 as single channel from **Figure.28**.

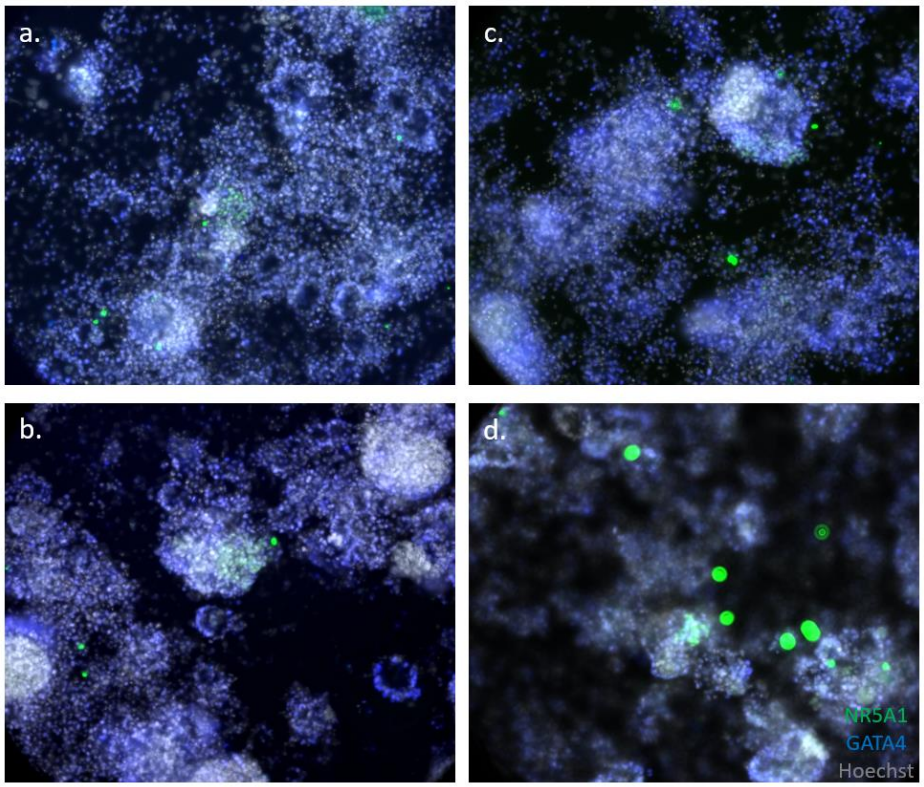


Figure.30. IF staining for NR5A1, GATA4 and Hoechst from cells plated in **figure 25.b;** cells were analysed after 7 days on the ECM plates with ARPMI medium. **a**. Cells plated on FN1. **b**. Cells plated on VTN. **c**. Cells were plated on FN1+COL1+ Heparin. **d**. Cells were plated on TNC+COL1.

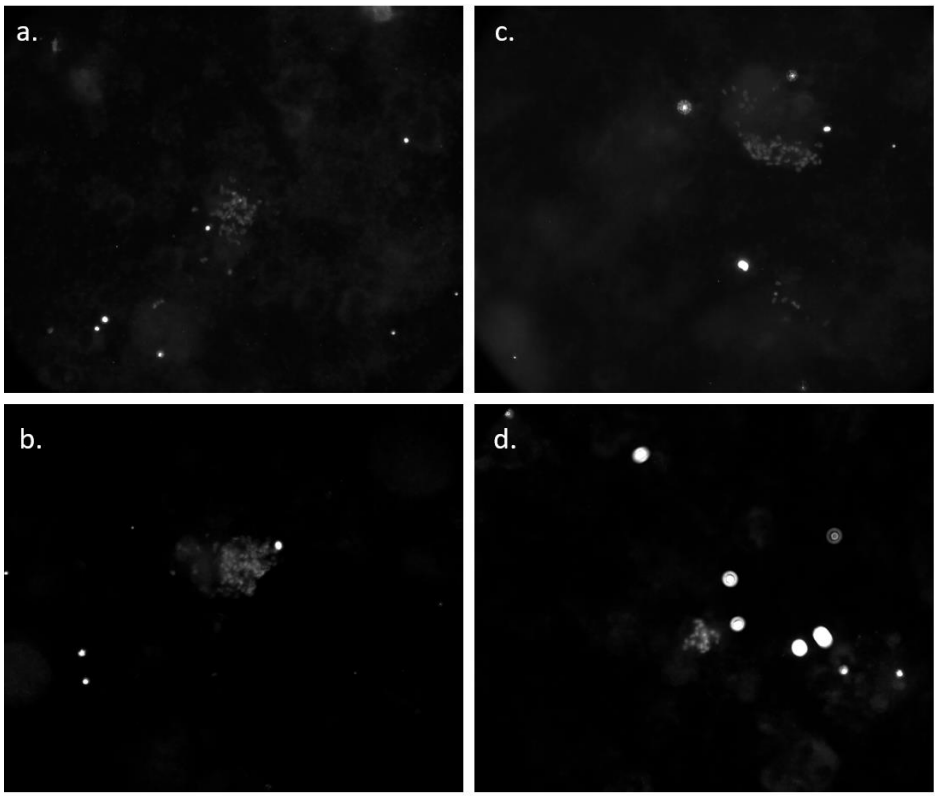


Figure.31. IF staining for NR5A1 as single channel from **Figure.30**

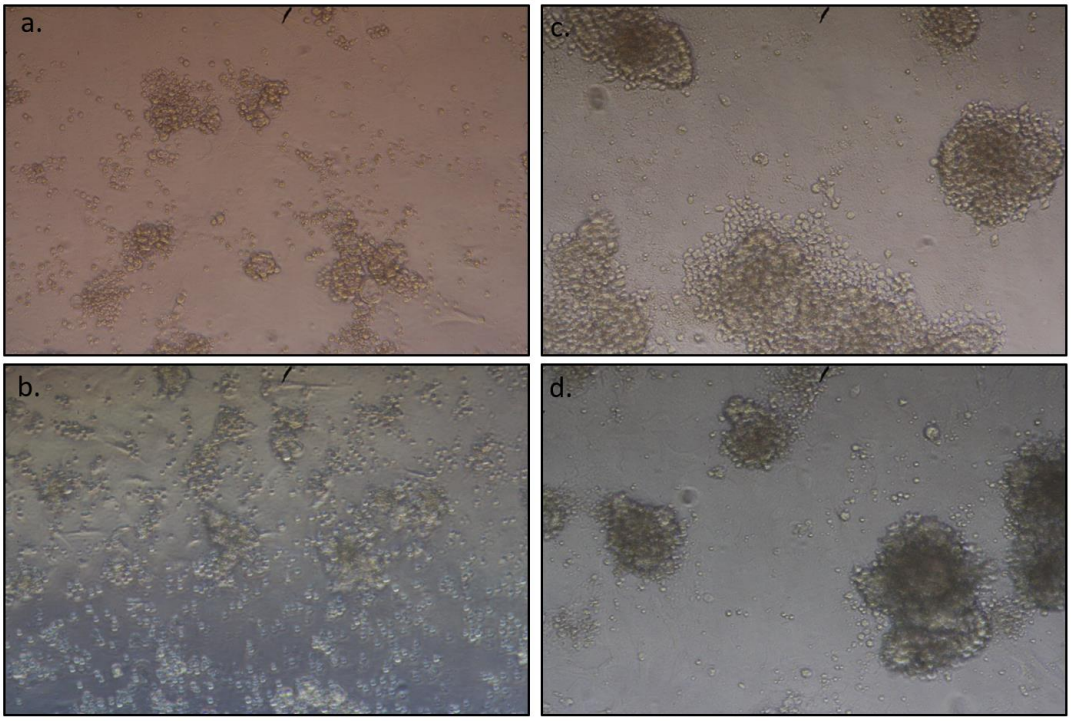


Figure.32. Brightfield images after two days of culture from cells plated in **figure 25.a** on Tenascin peptide-2 + Dextran ECM. **a&c** cells are growing in ADMEM-F12 and **b&d** in ARPMI. **c&d** cells were plated at their respective medium, with the addition of the medium they have been cultured before platting – M.CM (Mesodermal Conditioned Medium).

a manner resembling the **Figure.28b**, surrounded by GATA4 positive cells (**Figure.30,31)**. Samples in FN1 conditions exhibited the highest frequency of NR5A1 expression with 8 out of the 18 samples, which was more than double of VTN which had the second highest with 3/18. Although these results are encouraging, the percentage of NR5A1 expressing clones per positive sample accounted for less than 1% of the total colonies. In addition, the inconsistency of the appearance of positive colonies per sample indicate that the cells have the potential, but that additional optimization was required. Since during the transition to the new ECM there is cell death, we aimed to optimize this step to minimize stress for the cells.

2.3.b. Culture conditions optimization on FN1.

The use of conditioned medium is generally accepted as a mild way to wean off cells from a 'known' environment and introduce them to a new one (Caneparo et al., 2020; Furno et al., 2018; Jougasaki, 2010; Lincks et al., 1998). This was firstly tried on the commercially available plates of **Figure.25a,** using a condition of Tenascin peptide-2 + Dextran. We selected this condition since cells showed both the highest mortality, but also the highest number of NR5A1 positive clones. When transferring cells from the A.M. stage onto ECM plates, 25% of conditioned medium (the medium cells were grown in) was added. Two different media were also used ARPMI and ADMEM-F12, which are similar medium, but have minor differences regarding concentration of ingredients and a twice as high glucose concentration in ADMEM-F12 medium. The medium of DMEM-F12, RPMI, APEL2, K.O.-DMEM, DMEM and MEM were also tested together with ADMEM-F12 beforehand in a replication of a setup shown in **Figure.30**. In brief, only ADMEM-F12 resulted in NR5A1 positive colonies (data not shown). The effect of the M.CM (Mesodermal conditioned medium) on cell survival and morphology was striking (**Figure.32**). Importantly, the addition of M.CM did not have any negative effect on the generation of NR5A1^{+} colonies, but rather increased differentiation with up to ~5% of colonies being positive for the

steroidogenic marker. By applying the same method on the homemade ECM plates the percentage of NR5A1 colonies per positive sample increased to ~3% on FN1 coated wells, but there was not a significant increase on the positive samples (wells/plate). Since the increase of survivability did not answer the problem of the consistency, an additional signalling queue seems to be required for faithful differentiation of cells.

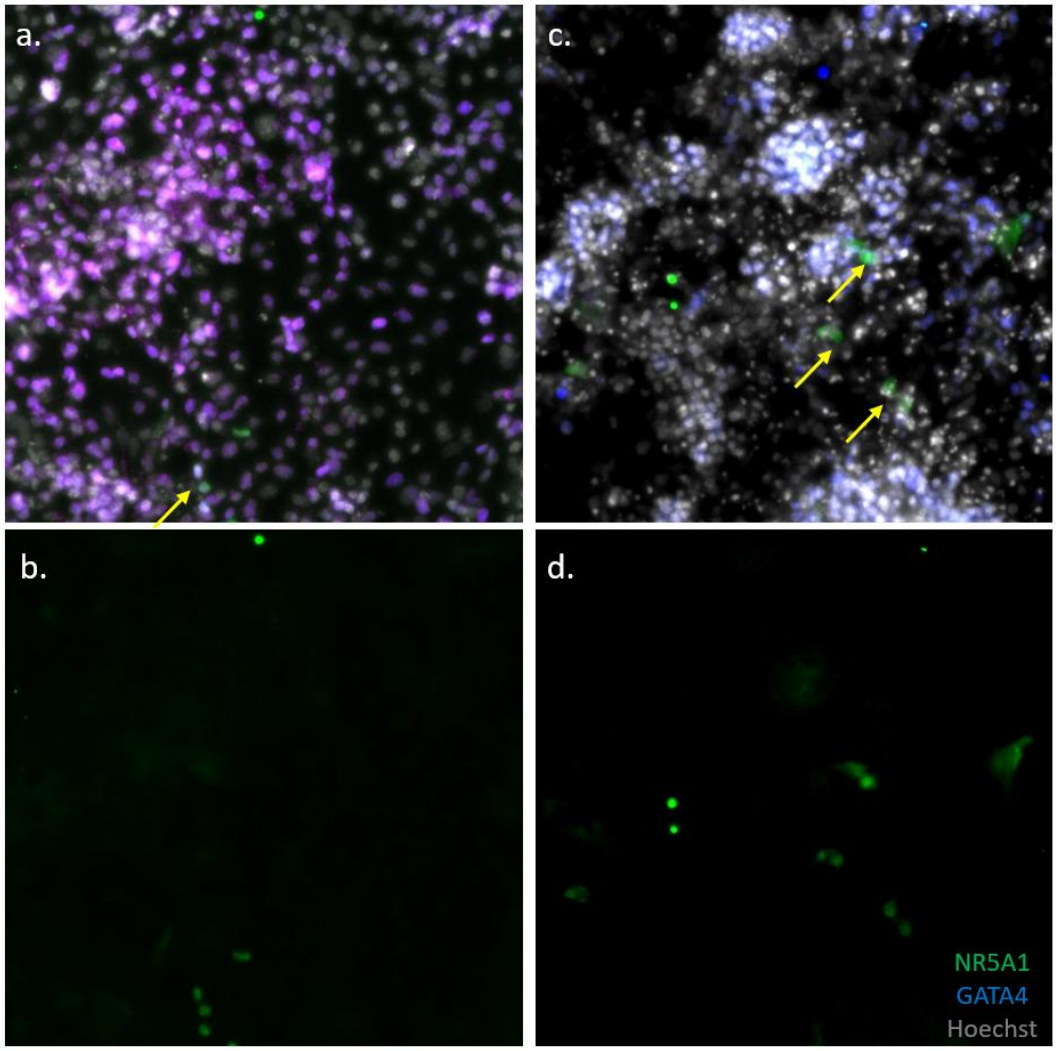


Figure.33. (**a&c**) IF staining for NR5A1, GATA4 and Hoechst from cells plated in **figure 25.b.** These cells have been growing for either 2 days in ADMEM-F12 (**a&b**) or ARPMI (**c&d**) medium with the addition of 25% M.CM on FN1 coated wells. Arrows indicate NR5A1 specific nuclear staining. (**b&d**) IF staining for NR5A1 as single channel.

To effectively improve the consistency of the NR5A1 colonies, we had to investigate when the earliest point is when NR5A1 positive cells appear in the culture. By having this information, a future treatment could be administrated to improve the efficiency, but not to disturb the fating of the cells. FN1 was chosen as the ECM with the highest potential for this differentiation protocol, due to the higher frequency and percentage of NR5A1 positive colonies per well. Once again, using the same method, a time-course screening experiment was done, while also checking how long the M.CM could be necessary for this event. IF was performed for NR5A1 and GATA4 (**Figure.33**). In both medium the earliest point and condition of the first NR5A1 positive cells was after two days with M.CM, without any change of the initial platting medium. Most of the cells in the culture were expressing GATA4, a finding expected based on the RT-qPCR results of the A.M. stage (**Figure.11b,c**). In their majority, the

cells in ARPMI had already formed colonies, in comparison with the ADMEM-F12 where F12 where colony formation was less advanced. NR5A1 positive cells were found either isolated or as a part of a GATA4 positive colony. Unfortunately, without any live imaging or with a tracing tool for those NR5A1 positive cells, we cannot know if these proliferate and thus creating a colony or the GATA4 positive cells start expressing NR5A1 or both.

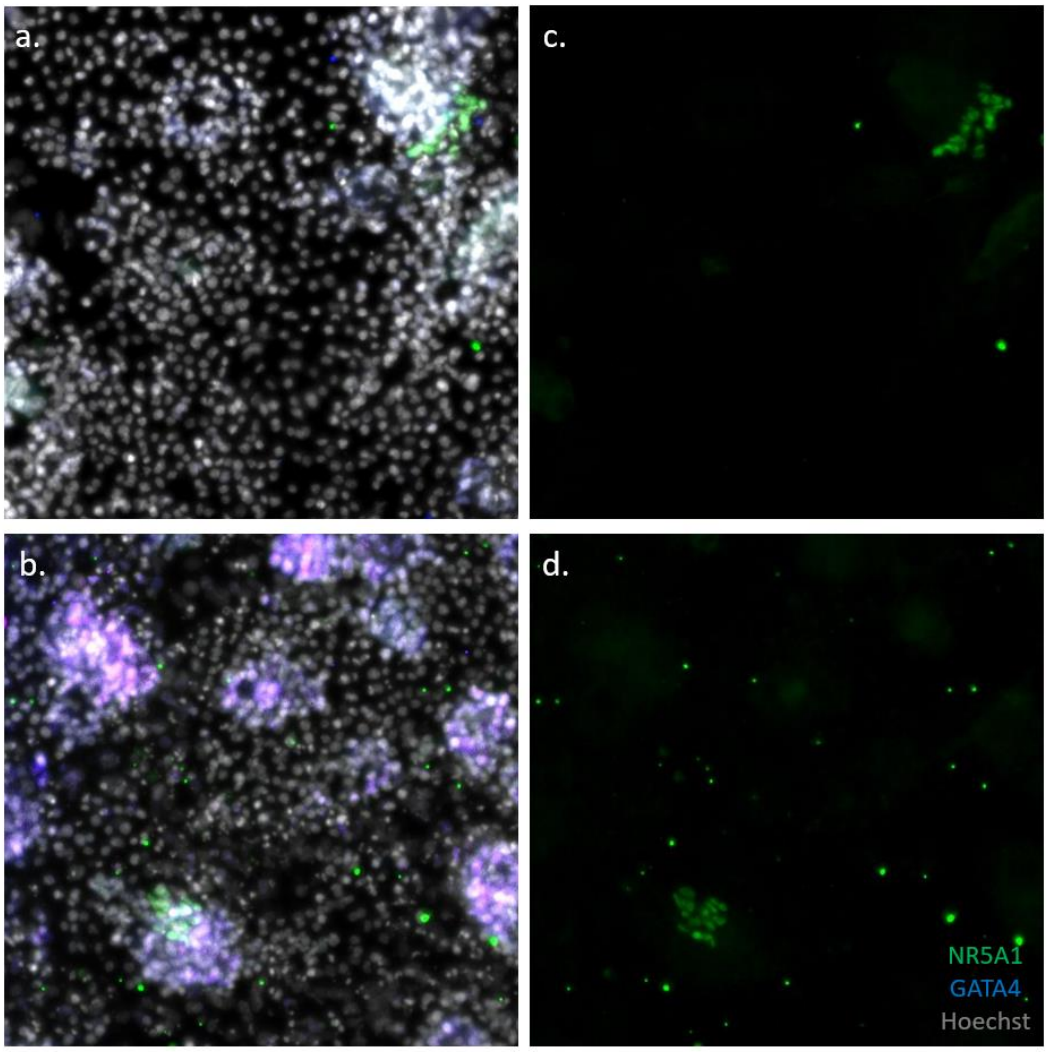


Figure.34. IF staining for NR5A1, GATA4 and Hoechst from cells plated in **figure 25.b.** These cells have been growing for 4 days with either 2 days of 25% M.CM addition (**b&d**), on plating, or without (**a&c**) in ADMEM-F12 on FN1 coated wells. (**c&d**) IF staining for NR5A1 as single channel.

A similar trend followed up also after 4 days. In both medium, without the presence of M.CM there were less NR5A1 positive colonies per samples, something that may be linked with the reduced population of GATA4 positive cells in the culture after 4 days (**Figure.34-35a,c**). Another observation one can make is that in both conditions of ARPMI, colonies were formed in larger clusters and at higher frequency (**Figure.35**) than those of the ADMEM-F12; especially in the non-M.CM condition (**Figure.34**). We can also see that the GATA4 positive cells are predominantly located in the clusters, while there are cells outside of the colonies. These cells are creating a carpet like layer when seen under brightfield and we speculate that they are endothelial cells, which would explain the observed *Sox17* expression described above. Indeed, it has been reported that FN1 supports endothelial cell growth and vascularization (X. Zhou et al., 2008). Nevertheless, after 4 days on FN1 coated plates there

was an emerge of NR5A1 positive colonies. In general, the ADMEM-F12 seems to have a delayed differentiation, since the cells growing in this medium, they are delayed on the colony formation at every step. Therefore, ARPMI is chosen as the most suited medium out of the two to be used in the next experiments. An additional note though has to be made for **Figure.35a** where a NR5A1 positive colony can be seen as a part of a bigger colony, but it is not surrounded by cells expressing GATA4 or co-expressing it in NR5A1^{+} cells.

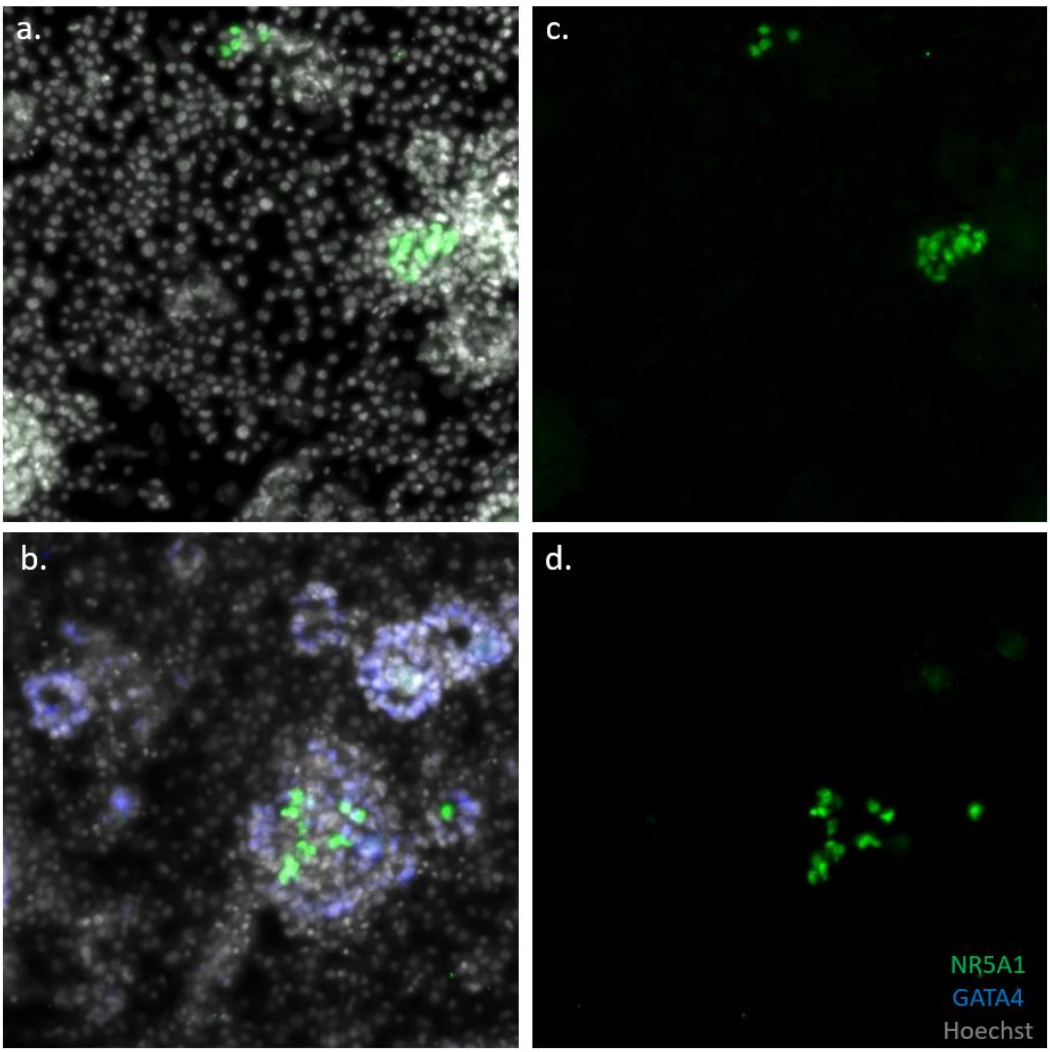


Figure.35. IF staining for NR5A1, GATA4 and Hoechst from cells plated in **figure 25.b.** These cells have been growing for 4 days with either 2 days of 25% M.CM addition (**b&d**), on plating, or without (**a&c**) in ARPMI on FN1 coated wells. (**c&d**) IF staining for NR5A1 as single channel.

In summary, we have seen that NR5A1 expressing cells appear preferentially on FN1 ECM and that the use of conditioned medium reduces stress when transitioning cells from Geltrex to FN1. We have also learnt that for proper fating of cells ARPMI was superior out of the medium we have tried this far. A further optimization has to be done to increase the consistency and efficiency of the protocol. The intervention (when to start the treatments) was pointed out to be optimal in the approximate window of 2 days on the ECM, since at that stage we observe the first NR5A1 positive cells, and it can influence their further fating.

2.3.c. Pathway screening for steroidogenic fating of the cells.

Subsequently, a timepoint induction experiment took place, where the samples were exposed to signalling ques for different lengths on time (0, 2 or 4 days) on FN1 coated plates. BMP/TGFβ signalling pathway was the first to be tested in these conditions, as it has been experimented in a large variety of related differentiation protocols (Knarston et al., 2020; Rore et al., 2021; Y. Yang et al., 2020). From spatiotemporal point of view the BMP pathway can affect the differentiation of this tissue, and it has been shown to have a pivotal role in adrenal medullary development (Saito et al., 2012; Saito & Takahashi, 2015). Additionally, we wanted to test the effect of PKA signaling on our cultures, as this pathway is known to induce *Nr5a1* expression of the adrenal cortex *in vivo* (Drelon et al., 2016) and *in vitro* (Kulcenty et al., 2015). Additionally, cAMP/PKA induction has been used in most of the direct differentiation to steroidogenic cells protocols and it worked in a replication of these experiment for us (page 54).

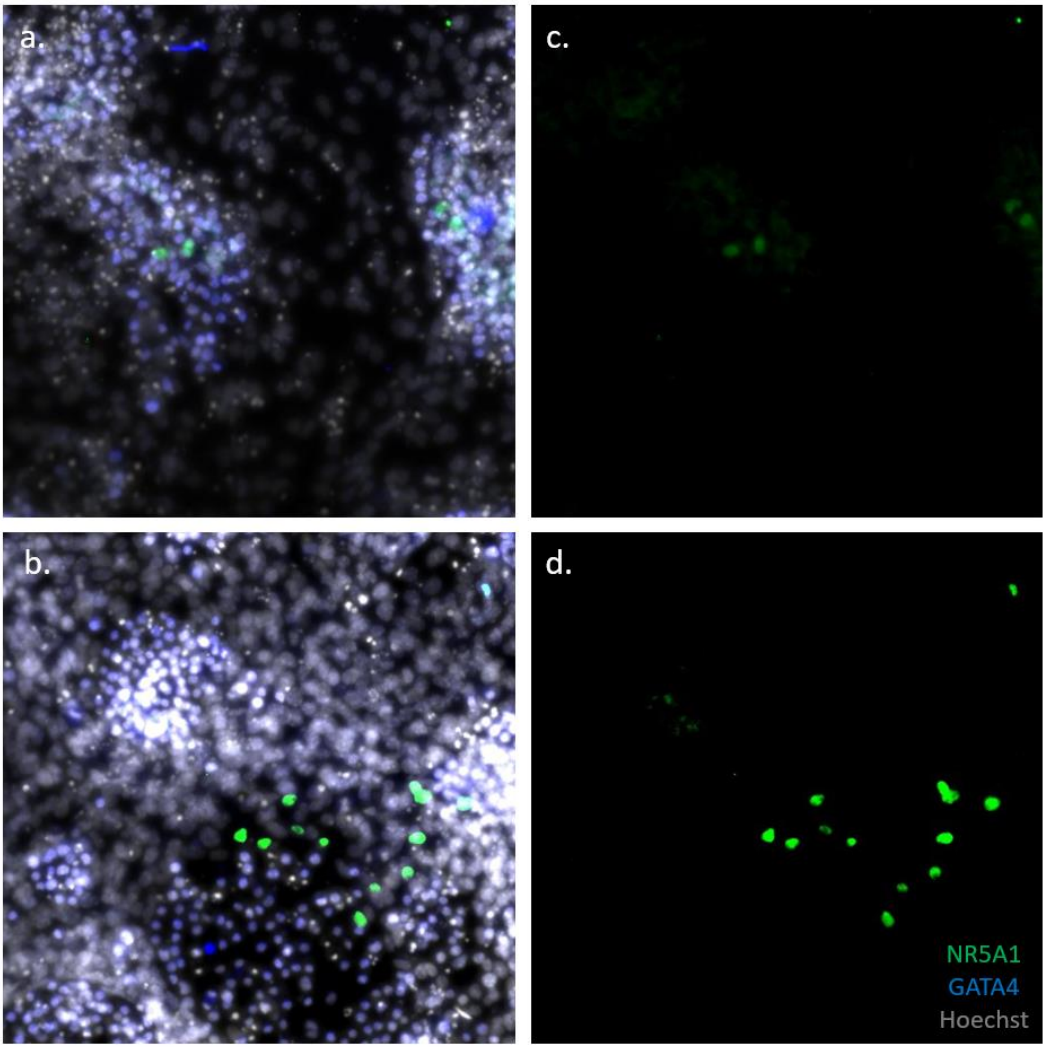


Figure.36. (**a&b**) IF staining for NR5A1, GATA4 and Hoechst from cells plated in **figure 25.b.** These cells have been growing for 4 days with 2 days of 25% M.CM addition, on plating, in ARPMI on FN1 coated wells; and treated at the same time as platting them. (**a&c**) 100nM 8-Br-cAMP + 50ng/ml Noggin, (**b&d**) 100nM 8-Br-cAMP + 100ng/ml BMP4. (**c&d**) IF staining for NR5A1 as single channel.

A thorough screening for different concentrations of Noggin (the physiological antagonist of BMP signalling), BMP4 (a major inductor of BMP pathway), DMH1 (mainly an ALK2 inhibitor), SB431542 (a

selective inhibitor of ALK4/5/7) and Dorsomorphin (a potent inhibitor of ALK2/3/6 and AMPK), with or without the addition of 100nM 8-Br-cAMP (activator of PKA signalling) was carried out. DMH1 did not show any benefit for the cultured cells (data not shown). It was quickly apparent that the treatment of the cells on the day of plating with the M.CM, did not benefit them (**Figure.36**). The only cultures that had some cells expressing NR5A1 were those treated with Noggin and BMP4, in the presence of 8-Br-cAMP (**Figure.36**), and they were not in colonies. On the other hand, when cells were treated after the initial two days with M.CM containing medium, we could find NR5A1 expressing cells in all conditions; indicating that this is a more proper timing for the treatments. Furthermore, cultures with the chemical inhibitors of ALK receptors and 8-Br-cAMP showed some nicely formed colonies (**Figure.37** suggesting that 8-Br-cAMP is indeed beneficial for NR5A1 expression. Another possible conclusion from this experiment is that fine tuning of BMP/TGFβ signalling pathway may be necessary for increased sufficiency and reproducibility of cells differentiating toward AGP, in this protocol.

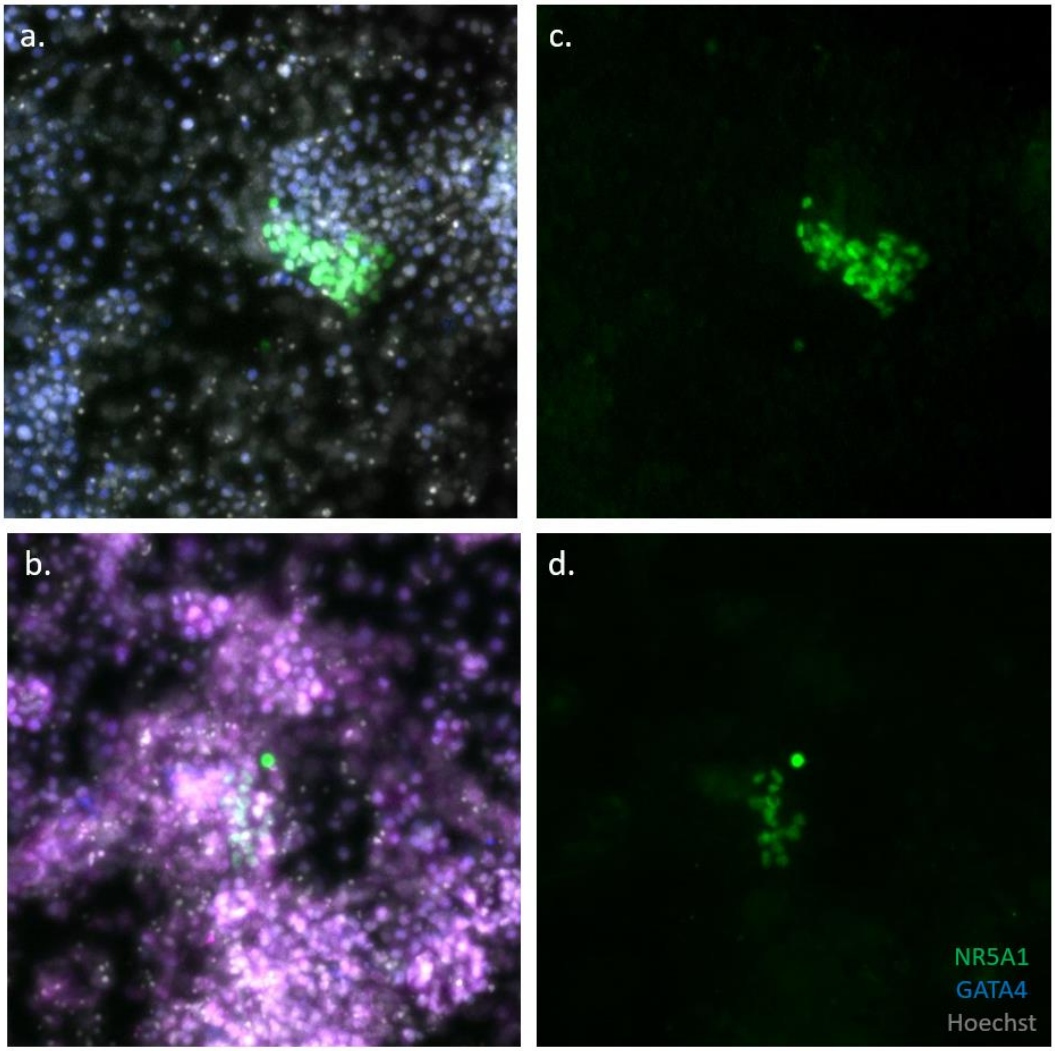


Figure.37. (**a&b**) IF staining for NR5A1, GATA4 and Hoechst from cells plated in **figure 25.b.** These cells have been growing for 4 days with 2 days of 25% M.CM addition, on plating, in ARPMI on FN1 coated wells; and treated for 2 days after the 2 days of initial platting with M.CM containing medium (**a&c**) 100nM (**c,f**)+ 0.5 SB431542, (**b&d**) 100nM 8-Br-cAMP + 50ng/ml Dorsomorphin. (**c&d**) IF staining for NR5A1 as single channel.

We next aimed to further decipher the cues leading to NR5A1 expression and thus the initiation of a steroidogenic profile in these cells. ARPMI has a component called AlbuMAX II, which is a serum-like ingredient rich in lipids and albumin, and we hypothesized that this component may lead to a higher degree of variability during the differentiation process. E6 medium is a basic medium widely used in hIPSCs differentiation protocols, as it allows a greater degree of plasticity, while providing the necessary ingredients for cell differentiation. Recalling the trials with ADMEM-F12, we wanted to use a medium that resembles ARPMI, especially in terms of glucose concentration, as this was a likely factor enhancing differentiation. Thus, a homemade E6 (cE6) medium was prepared with the basis of Ham's F-12 Nutrient Mix, which glucose concentration is closer to ARPMI. BMP4 and Noggin, in concentrations defined by the previous experiment were used in this trial, with or without the addition of 8-Br-cAMP. Additionally, in this experiment we used an alternate patterning of the media between plating and treating the cells. Cells were plated with either cE6 or ARPMI medium, then after the two days of M.CM the cells were treated either at the same medium that they were cultured so far, or their medium was switched and then treated in the new medium. The treatment lasted for two days and after the cells were let on a plain refreshed medium, the same which was used during their treatment, for another 3 days (**Figure.s5**).

In agreement with previous experiments, conditions with 8-Br-cAMP showed consistently higher numbers of NR5A1 expressing clones (**Figure.38**). ARPMI-ARPMI conditions were comparable with previous experiments, and most of the positive colonies were found in the 8-Br-cAMP+Noggin condition. By contrast, cE6-cE6+ 8-Br-cAMP showed the appearance of NR5A1 positive cells in 85% of samples, with approximately ~2% positive colonies on average. The most successful condition was media containing BMP4 (**Figure.38a,d**), whereas cells did not seem happy in the presence of Noggin (**Figure.38c,f**). A similar observation was made for ARPMI-cE6 treatment, but the overall number of positive colonies was lower (**Figure.38b,e**). Culturing under cE6-ARPMI conditions was least successful and no positive colony could be identified. It has now become clear that the presence of cAMP greatly boosts the potential by generating bigger colonies of NR5A1 positive cells at a higher frequency. Furthermore, from the latest results we can see that in the presence of ARPMI medium, conditions where TGFβ signalling is inhibited give more favourable results. The opposite can be attributed to the cE6 medium conditions where the worst results were observed after Noggin treatment, whereas it seems BMP4 boosted the steroidogenic fate of cells. This observation may indicate that the traces of TGFβ signalling in ARPMI are more than enough for proper differentiation, whereas inhibiting this pathway in cE6 is counterproductive. Finally, SOX17 staining was observed frequently together with GATA4, which may indicate a more cardiac fate of cells (Saba et al., 2019).

Based on the results we decided to use either ARPMI or cE6 medium without switching between them. Since there was always better results under 8-Br-cAMP treatment, we investigated if this treatment was enough to boost the fating of the cells. Thus, the previous experiment was replicated using the most prominent conditions: Noggin or BMP4 with 8-Br-cAMP or 8-Br-cAMP alone, in cE6 or ARPMI medium for two days. In this experiment we also wanted to further investigate the identity of other cells surrounding the NR5A1 positive cells. Therefore, samples were stained for WT1 and SOX2 expression, in the addition of NR5A1 and GATA4 (**Figure.39-41**). As expected from previous results, in ARPMI the best condition was with Noggin + 8-Br-cAMP (**Figure.40**), although 8-Br-cAMP treatment alone was sufficient to produce NR5A1 positive colonies. In cE6 media, Noggin was not enhancing the differentiation of cells, while a combination of BMP4 and 8-Br-cAMP or 8-Br-cAMP alone was promoting the NR5A1 expression (**Figure.39,41**). Interestingly, in cE6 medium conditions, the majority

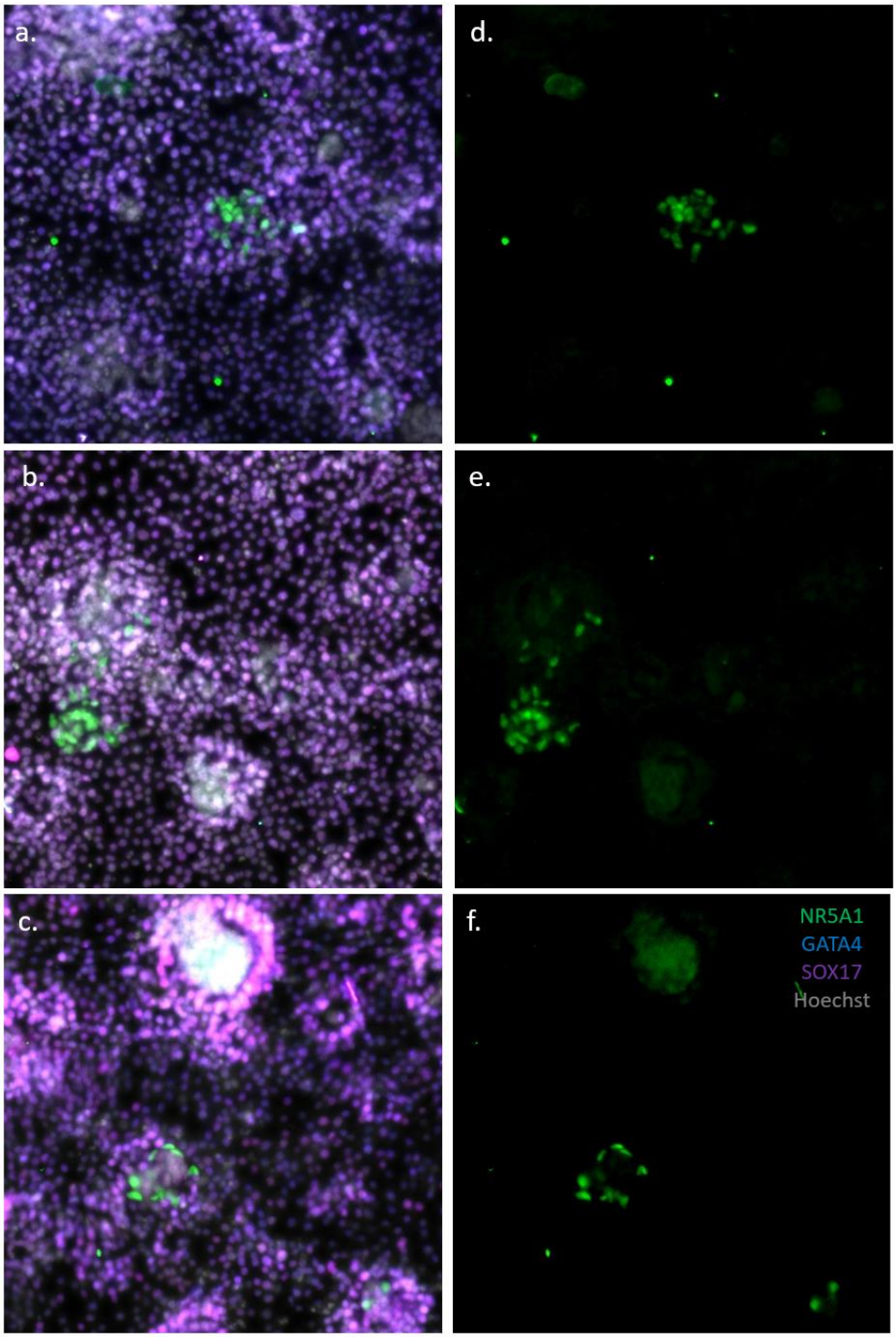


Figure.38. (**a,b,c**) IF staining for NR5A1, GATA4, SOX17 and Hoechst from cells plated in **figure 25.b.** These cells have been growing for 7 days with 2 days of 25% M.CM addition, on plating, in ARPMI (**b,e**) or cE6 (**a,c,d,f**) medium on FN1 coated wells. They switch medium to cE6 and then treated for 2 days with 100nM 8-Br-cAMP + 100ng/ml BMP4 (**a,b,d,e**) or + 50ng/ml Noggin (**c,f**), after the 2 days of initial platting with medium containing M.CM. The cells were thereafter cultivated with their respective plain medium. (**d,e,f**) IF staining for NR5A1 as single channel.

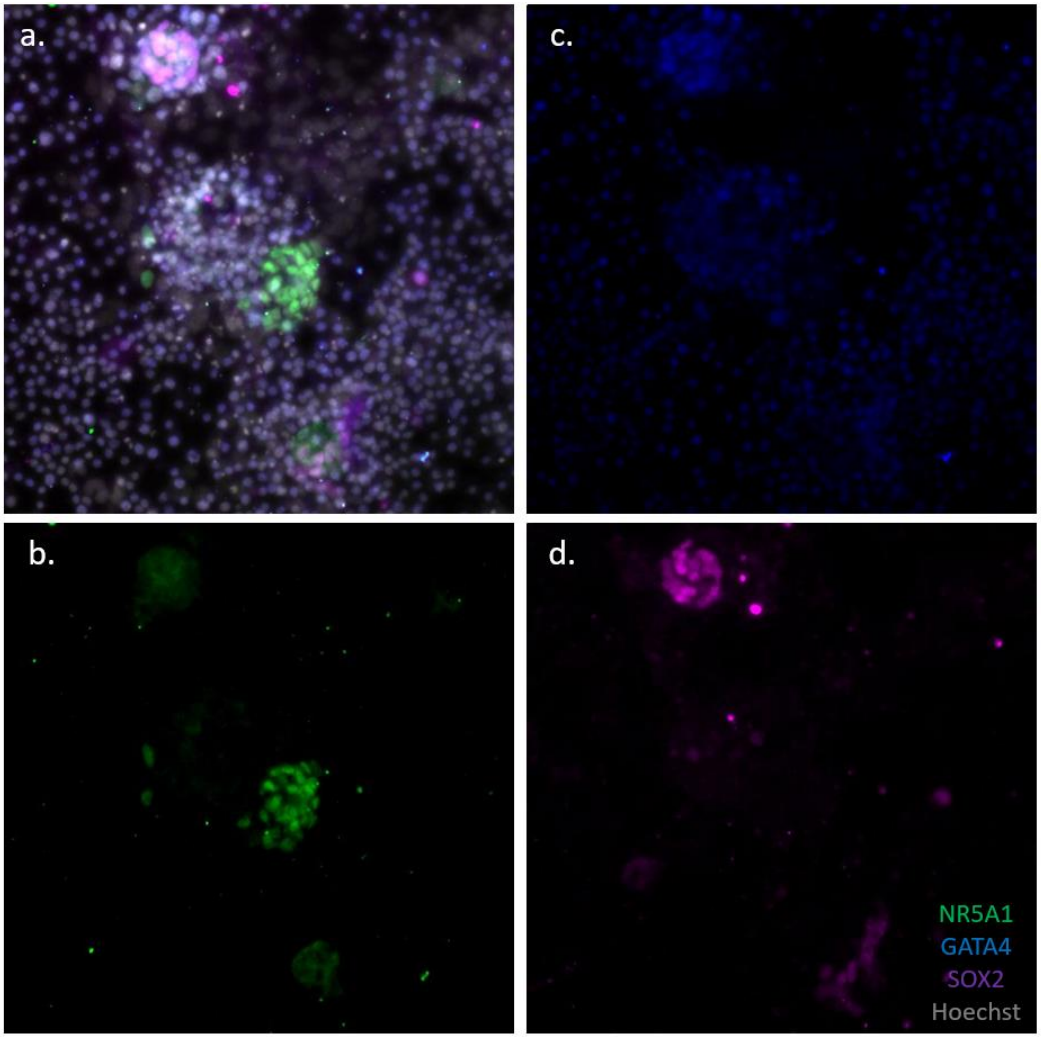


Figure.39. IF staining for NR5A1, GATA4, SOX2 and Hoechst from cells plated in **figure 25.b.** These cells have been growing for 7 days with 2 days of 25% M.CM addition, on plating in cE6 medium on FN1 coated wells; and treated for 2 days with 100nM 8-Br-cAMP + 100ng/ml BMP4, after the 2 days of initial platting with M.CM containing cE6 medium. IF staining for NR5A1 (**b**), GATA4 (**c**) and SOX2 (**d**) as single channels.

of NR5A1 colonies were in the proximity of a SOX2-GATA4 positive colonies. A possible explanation for the SOX2 colonies is that they are epithelial of cells gastrointestinal (gut and stomach) identity (DeLaForest et al., 2021; Sankoda et al., 2021). Alternatively, they may represent an *in vitro* phenotype, since in previous steps of the differentiation we did not observe many cells of endodermal lineage. Furthermore, 8-Br-cAMP can promote other tissue lineages beside the steroidogenic ones (Ikuno et al., 2017; Kabeya et al., 2018; Ogawa et al., 2013), thus a further optimization to restrict differentiation will be needed at this step of our protocol.

In the follow up series, experiments were scaled up on 96well and 24well plates (**Figure.25c**) and in addition to 8-Br-cAMP additional factors were tested. FGF9 was used as it is a common growth factor utilized in *in vitro* differentiation experiments that aim at the steroidogenic lineage (Knarston et al., 2020; Rore et al., 2021; Seol et al., 2018). In addition, we wanted to test the Hh pathway, as SHH addition had shown promising results in a previous set of experiments. Moreover, gut epithelia produce SHH, and we speculated that the SOX2/GATA4 positive cells described above may have been a source of endogenous SHH. Instead of recombinant SHH, we opted for Purmorphamine, a small

molecule activating this signalling pathway. Thus, the samples were treated with different combinations of either FGF9, Purmorphamine (purmo) and 8-Br-cAMP, in cE6 or ARPMI. A summary of the conditions can be seen in Figure.46. Additionally, in this experiment, the samples were treated for a total for five days instead of two, as this appeared to provide better results when cultured in 3D (see below).

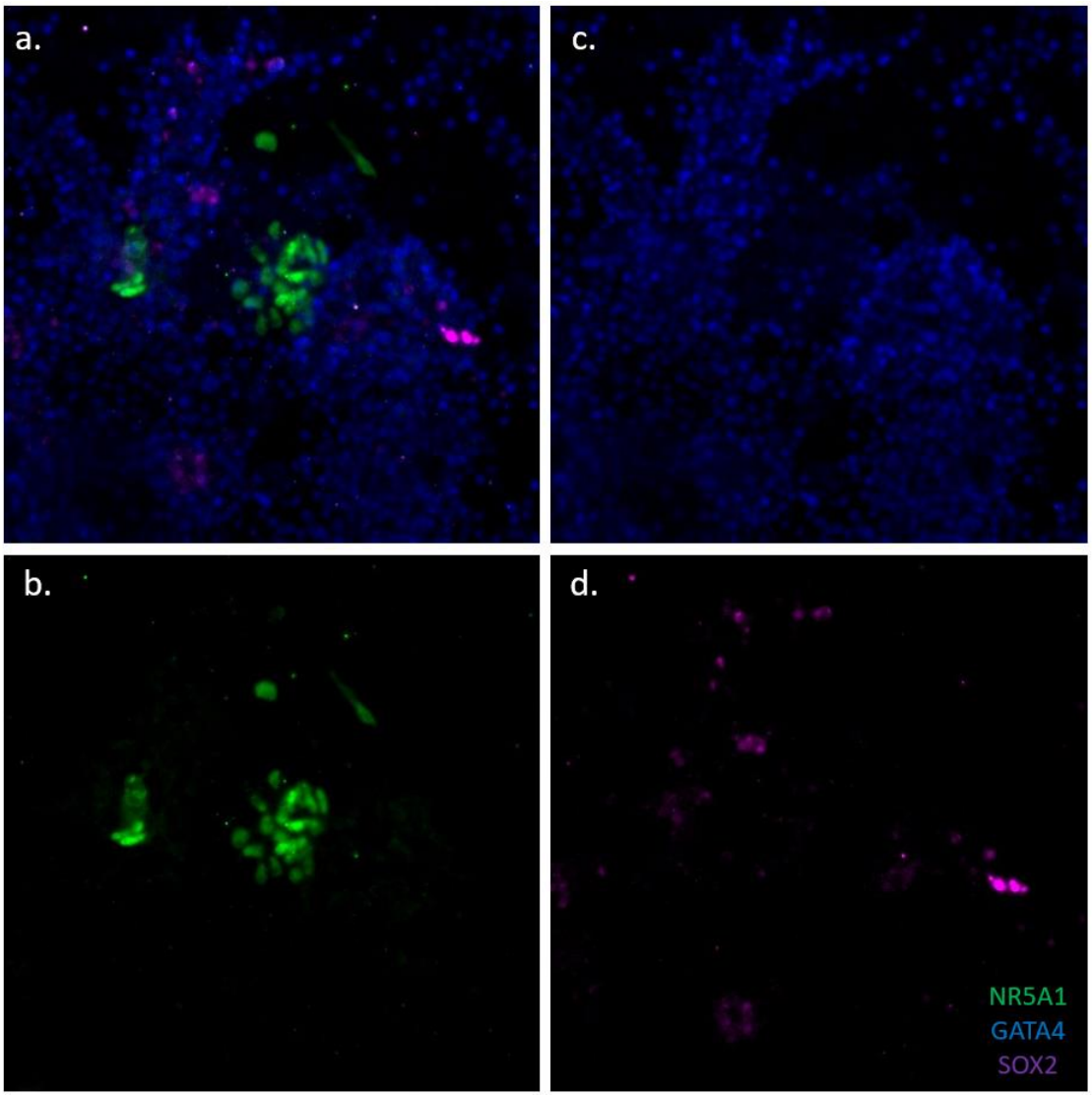


Figure.40. IF staining for NR5A1, GATA4 and SOX2 from cells plated in **figure 25.b.** These cells have been growing for 7 days with 2 days of 25% M.CM addition, on plating in ARPMI medium on FN1 coated wells; and treated for 2 days with 100nM 8-Br-cAMP + 50ng/ml Noggin, after the 2 days of initial platting with M.CM containing ARPMI medium. IF staining for NR5A1 (**b**), GATA4 (**c**) and SOX2 (**d**) as single channels. Hoechst was not applicable in these conditions due to technical difficulty.

Surprisingly, none of the E6 medium conditions yielded any NR5A1 positive cells, completely contradicting our previous observations for the 8-Br-cAMP treatment. One possible explanation is that PKA pathway activation only acts positively on the already fated cells and cannot be used as an early trigger of differentiation. Alternatively, 8-Br-cAMP prolonged treatment may not be beneficial in the E6 media. By contrast, conditions with ARPMI resulted in a high number of NR5A1 positive clones (**Figure.42-45**). All conditions with 8-Br-cAMP alone or with the addition of either FGF9 or purmo

resulted in NR5A1 expressing cells and colonies. The highest number of positive clones was observed under the condition ARMPI + 8-Br-cAMP alone, which yielded ~5% of positive colonies. The rest of the conditions did not give any NR5A1 expressing cells, which contrasts with our previous observations where plain media was sufficient to obtain a small population of NR5A1 positive cells. Addition of FGF9 appeared to suppress SOX2 positive colonies in the proximity of NR5A1, and positive cells failed to form more packed colonies (**Figure.42)**. The lack of SOX2 positive cells was observed also with the addition of purmo, but in this case the positive cells could form big colonies (**Figure.45)**. Both of these conditions yielded NR5A1 positive colonies in the range of 1.5-2%.

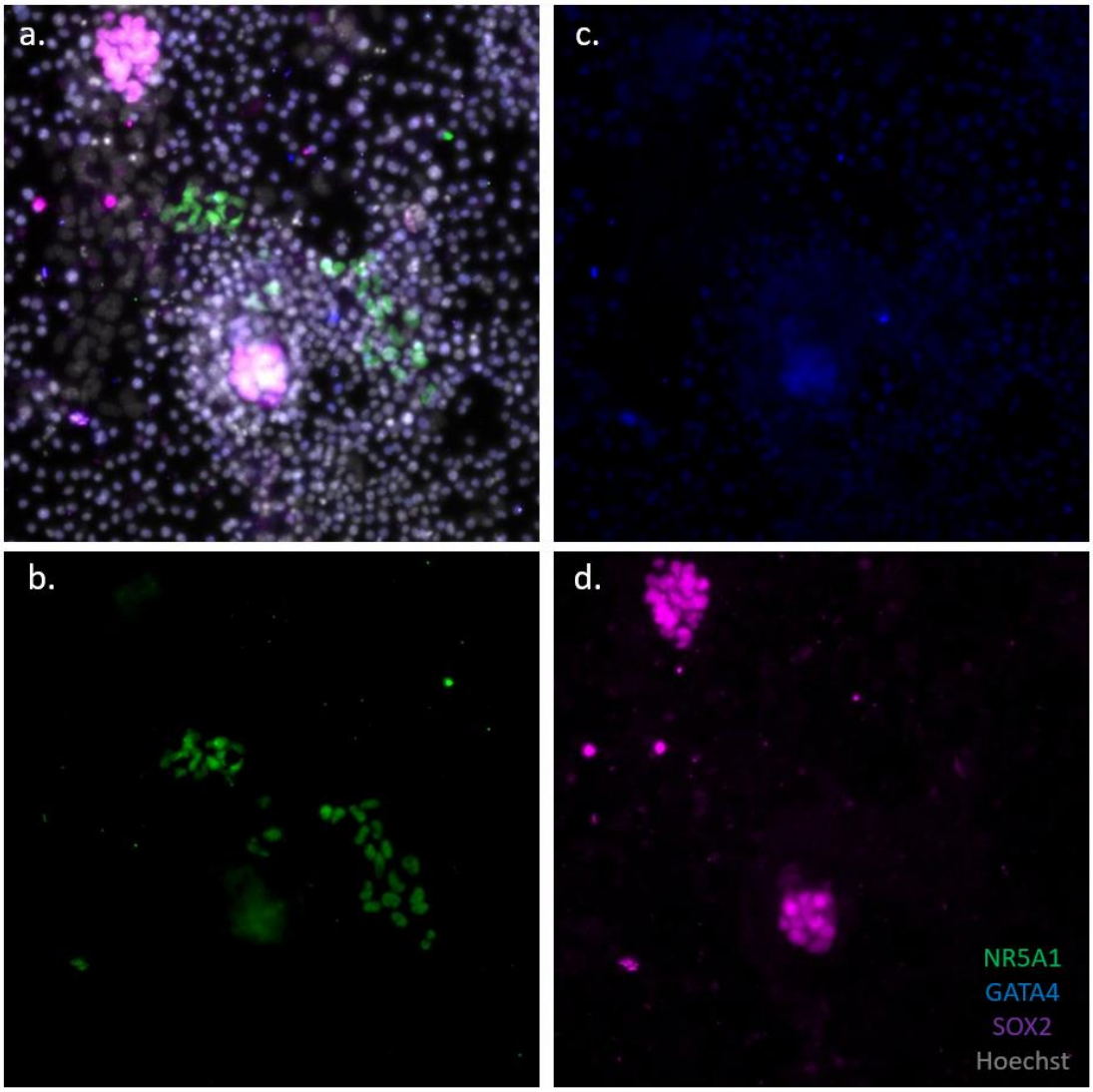


Figure.41. IF staining for NR5A1, GATA4, SOX2 and Hoechst from cells plated in **figure 25.b.** These cells have been growing for 7 days with 2 days of 25% M.CM addition, on plating in cE6 medium on FN1 coated wells; and treated for 2 days with 100nM 8-Br-cAMP, after the 2 days of initial platting with M.CM containing cE6 medium. IF staining for NR5A1 (**b**), GATA4 (**c**) and SOX2 (**d**) as single channels.

When treated only with 8-Br-cAMP, a pattern regarding NR5A1 expressing cells became apparent (**Figure.43,44**). NR5A1 positive colonies were observed even in the absence of SOX2 expressing cells in their proximity (**Figure.44**), but these areas seemed relatively poor of cells forming clusters. By contrast, we observed bigger NR5A1 positive cells that appeared to express higher levels, when they were close to colonies co-expressing GATA4 and SOX2 (**Figure.43**). We can speculate that SOX2

positive colonies produce a gradient of factors that promote the growth of NR5A1 positive cells. A potential candidate for such a factor is SHH, when combining the outcome of the results. The lack of NR5A1 positive cells in E6 media and non-treated ARPMI condition was disappointing, given that these conditions had yielded positive clones in previous experiments. A possible explanation may be the that conditions have not yet been optimized for 24well-plate wells, since all our previous experiments with the change of ECM were done solely in 96well-plates. Another, more worrying explanation can be that this is a result of events which take place during the fating of the cells at earlier stages which we were not able to monitor correctly.

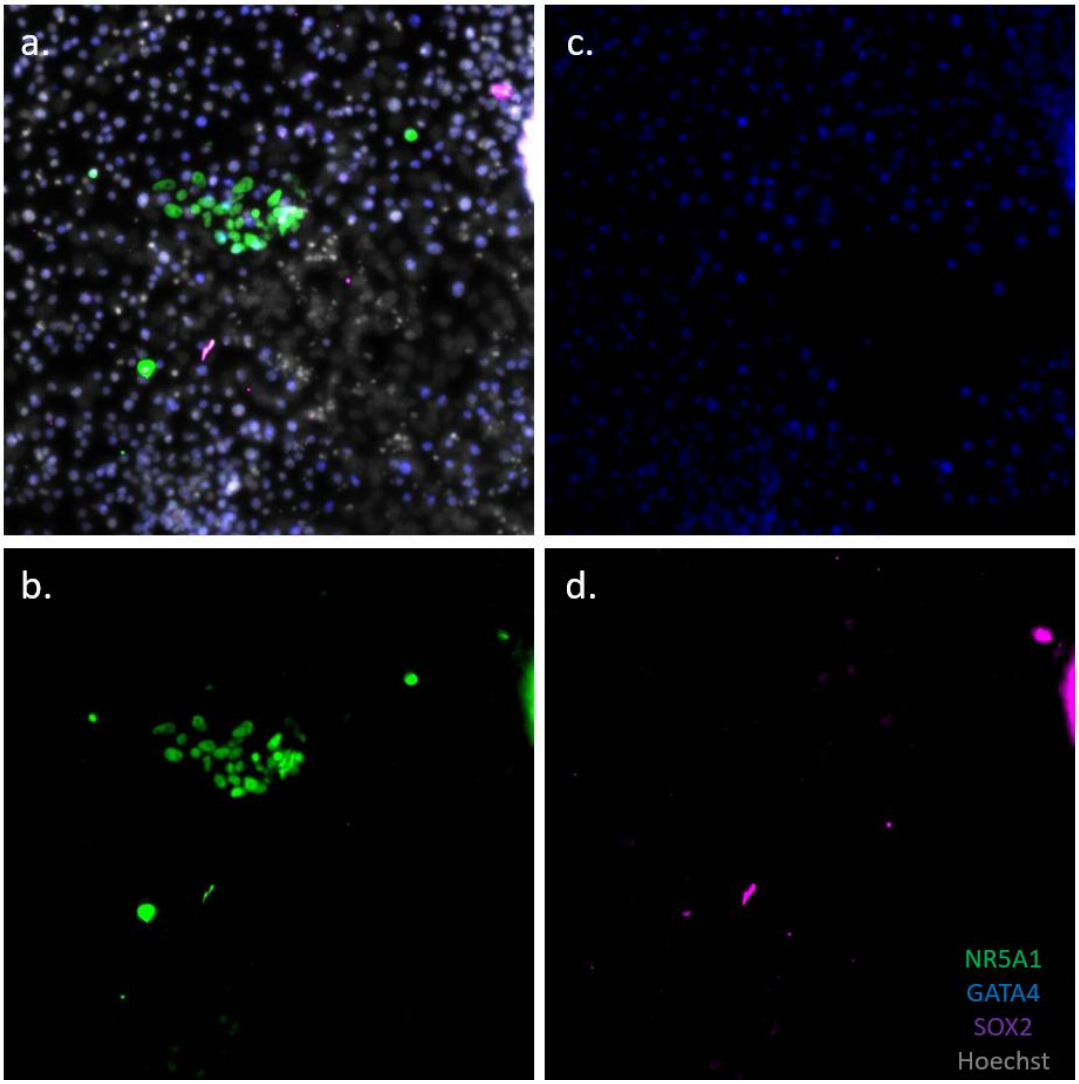


Figure.42. IF staining for NR5A1, GATA4, SOX2 and Hoechst from cells plated in **figure 25.c.** These cells have been growing for 7 days with 2 days of 25% M.CM addition, on plating in ARPMI medium on FN1 coated wells; and treated with 100nM 8-Br-cAMP + 100ng/ml FGF9, after the 2 days of initial platting with M.CM containing ARPMI medium. IF staining for NR5A1 (**b**), GATA4 (**c**) and SOX2 (**d**) as single channels.

In parallel to the above immunostaining, RT-qPCR analysis was performed to assess the expression levels of AGP markers and genes important for later steps of differentiation (**Figure.46**). In general, the expression of *Mc2r* was reduced after 7 days of treatment on FN1 and maintained only in ARPMI conditions (**Figure.46a**). Simultaneously, Nr5a1 expression was mainly upregulated in the conditions

were *Mc2r* was also, but in both media (**Figure.46b**). Combining both datasets, we can see that NR5A1 protein was only observed in samples were relatively high levels of *Mc2r* was expressed, coupled with *Nr5a1* upregulation (cAMP, cAMP+FGF9 and cAMP+purmo). Based on this experiment, there is an indication of a connection between *Nr5a1/ Mc2R* expression and NR5A1 being detected via IF. It seems that *Nr5a1* alone is not enough to boost the protein expression, but when there is also *Mc2R* upregulation NR5A1^{+} cells are present. This observation could mean a more mature state of cells, which is represented by the upregulation of *Mc2R*. An interesting note is that the cells of A.M stage expressed double the usual levels of *Mc2r* (**Figure.46a**) and *Wt1*.

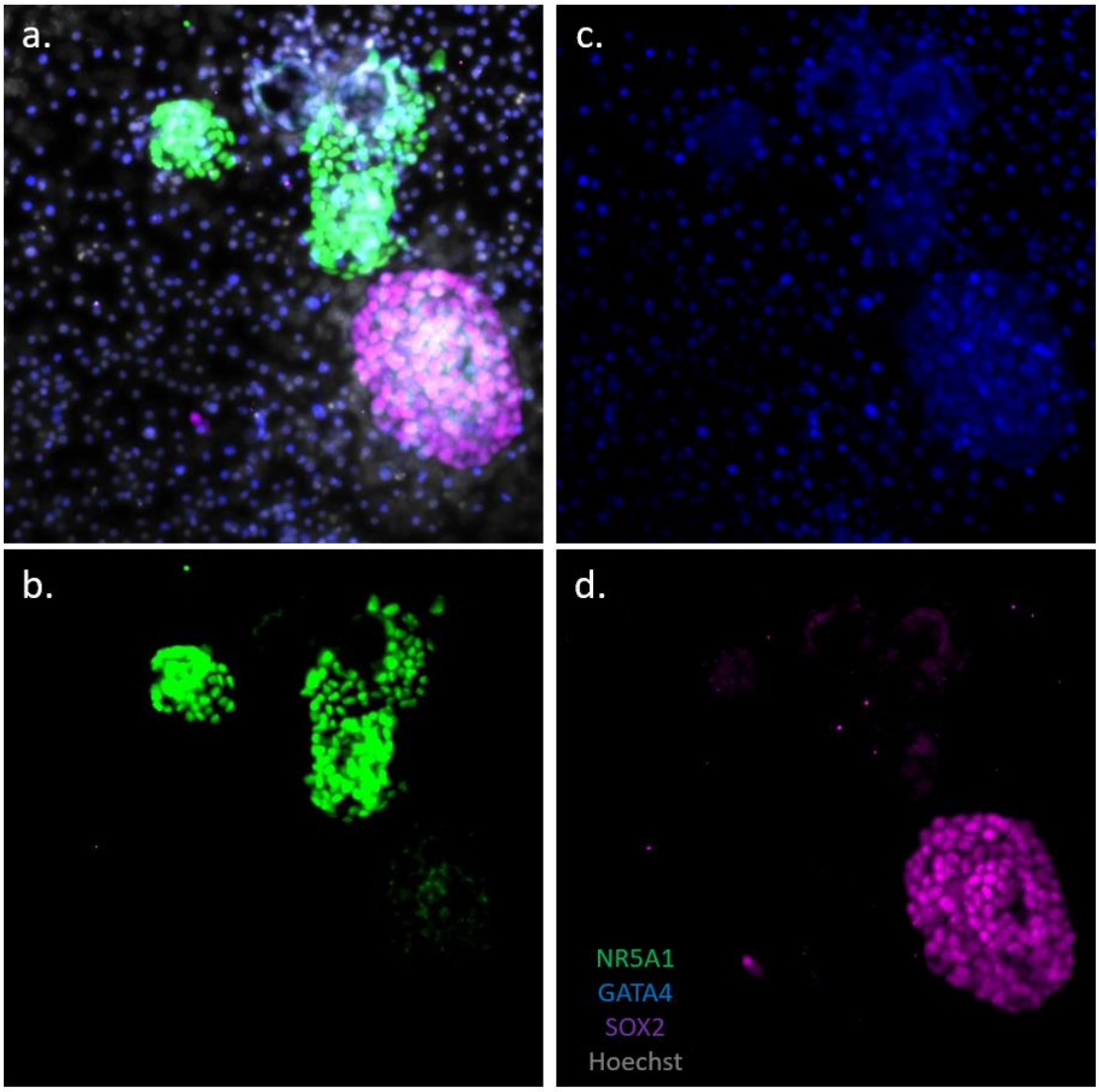


Figure.43. IF staining for NR5A1, GATA4, SOX2 and Hoechst from cells plated in **figure 25.c.** These cells have been growing for 7 days with 2 days of 25% M.CM addition, on plating in ARPMI medium on FN1 coated wells; and treated with 100nM 8-Br-cAMP, after the 2 days of initial platting with M.CM containing ARPMI medium. IF staining for NR5A1 (**b**), GATA4 (**c**) and SOX2 (**d**) as single channels.

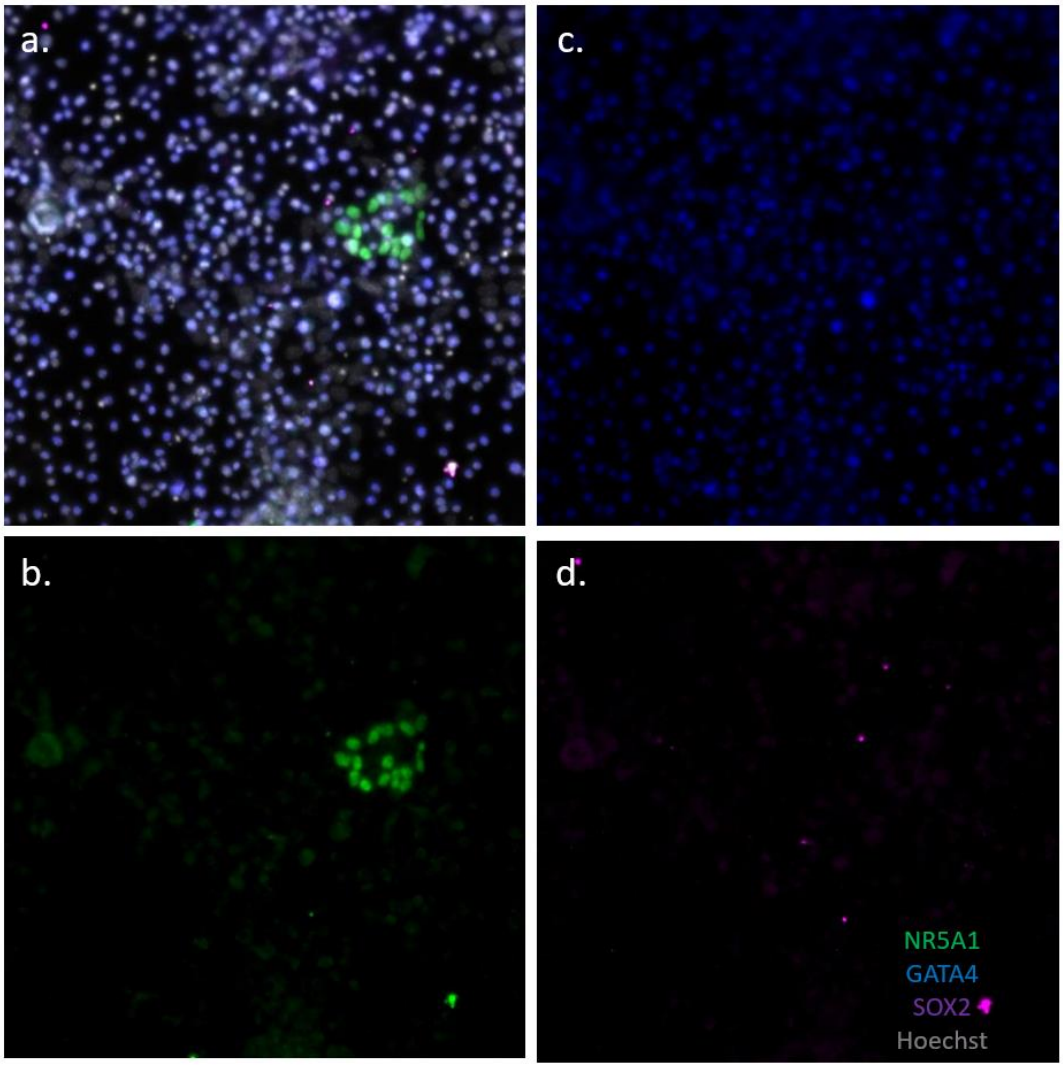


Figure.44. IF staining for NR5A1, GATA4, SOX2 and Hoechst from cells plated in **figure 25.c.** These cells have been growing for 7 days with 2 days of 25% M.CM addition, on plating in ARPMI medium on FN1 coated wells; and treated with 100nM 8-Br-cAMP, after the 2 days of initial platting with M.CM containing ARPMI medium. IF staining for NR5A1 (**b**), GATA4 (**c**) and SOX2 (**d**) as single channels.

2.4. Driving the steroidogenic fate through 3D assembly.

2.4.a. R1 cells.

Having gathered all these information and experience from the 2D ECM models, we sought to apply similar techniques to a 3D environment. The 3D approach has been shown in other systems to enhance differentiation by providing the correct niche. It can also be used to further dissect the mechanisms driving successful differentiation. A.M. cells were generated as described (**Figure.11a**). Once disassociated (50% Trypsin) they were placed in low attachment with either pyramidical (**Figure.47a**) or round (**Figure.47b**) bottom wells; the method of M.CM was also adopted for the 3D protocol. Furthermore, in order to minimise stress and disturbance of the aggregates, media were topped up (addition of fresh medium) and only changed after two days of culture. For further changes, half of the volume was replaced with fresh media. This method provides a smoother transition and may resemble more the *in vivo* situation, where there is always a gradient of factors.

We began the new series of 3D experiments by testing sphericalplates (Kugelmeier) (**Figure.47a**), which are providing a unique environment for the aggregates. Using these plates multiple aggregates of similar size are generated that can influence by each other through paracrine signaling. Based on our results from 3D models, we chose 8-Br-cAMP, RA (**Figure.24e,f**) and FGF9 (used in most AGP derived *in vitro* differentiation protocols) were tested. As media, ARPMI (**Figure.48a**) and cE6 (**Figure.48b**) were used. The choice of medium appeared to have dramatic consequences on the morphology of colonies. While aggregates grew in the centre of the microwells in ARPMI media, in cE6 media the aggregates not always properly formed, and the cells seem to attach or trying to migrate out of the well. This experiment was replicated two times with keeping 8-Br-cAMP and FGF9, but testing additional conditions in different media. Surprisingly, the same phenotype was not observed in the second replicate (Kug2), where cells were more spherical and showed no anarchic growth (data not shown). Similarly, in the third replicate (Kug3) the ARPMI and cE6 media no morphological difference was observed, but the aggregates were bigger and more expanded (**Figure.49**).

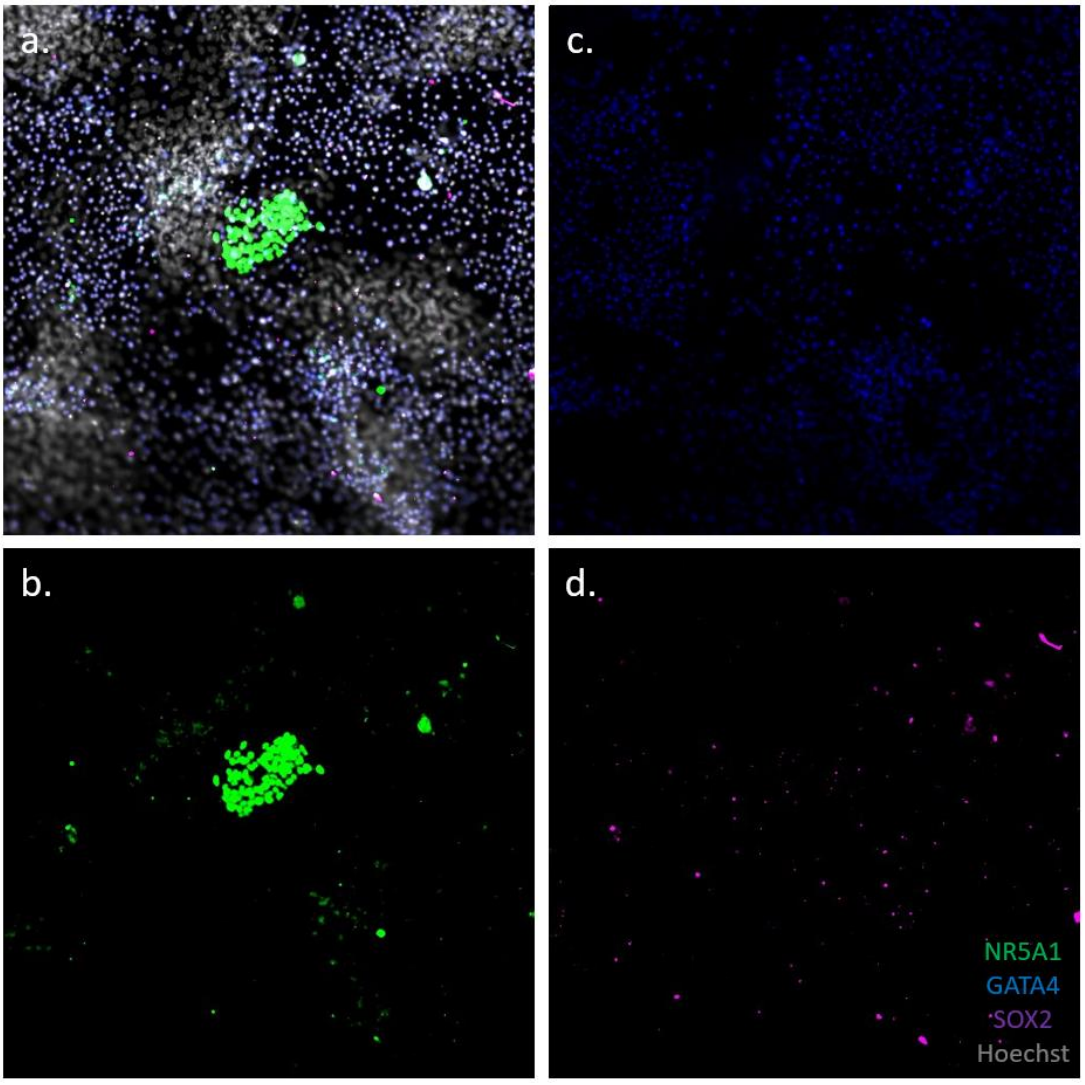


Figure.45. IF staining for NR5A1, GATA4, SOX2 and Hoechst from cells plated in **figure 25.c.** These cells have been growing for 7 days with 2 days of 25% M.CM addition, on plating in ARPMI medium on FN1 coated wells; and treated with 100nM 8-Br-cAMP + 1uM Purmorphamine, after the 2 days of initial platting with M.CM containing ARPMI medium. IF staining for NR5A1 (**b**), GATA4 (**c**) and SOX2 (**d**) as single channels. 10x magnification.

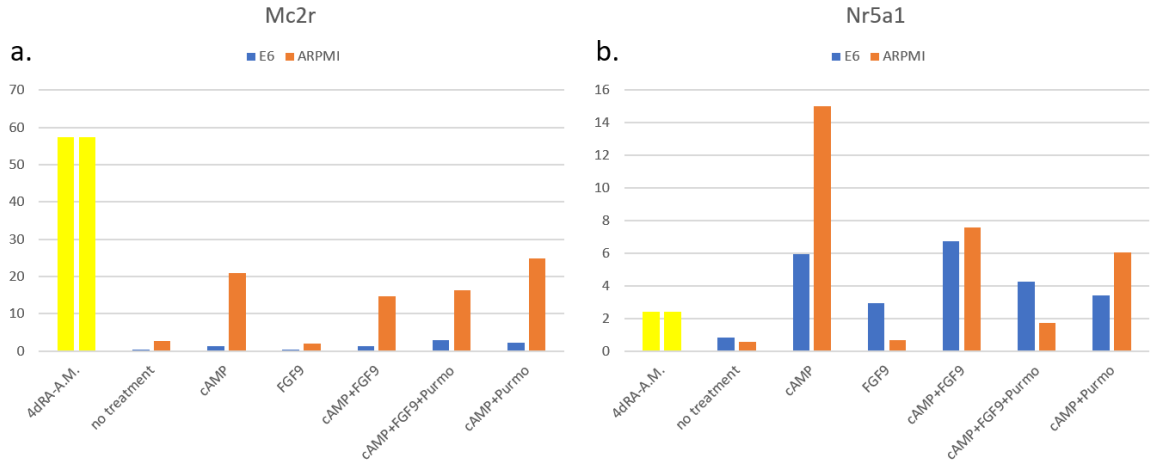


Figure.46. RT-qPCR from **Figure.42-45** duplicate experiment after 7 days of treatment as described, following the A.M stage**.** Relative expression of *Mc2r* (**a**) and Nr5a1 (**b**) to SDHA, compared with UR of E9.5. Combinations of 8-Br-cAMP (100nM), FGF9 (100ng/ml) and Purmorphamine (1uM) are shown on X axis, with blue bar corresponding to cells grown in cE6 medium and orange bar for ARPMI medium. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with *Sdha* expression.

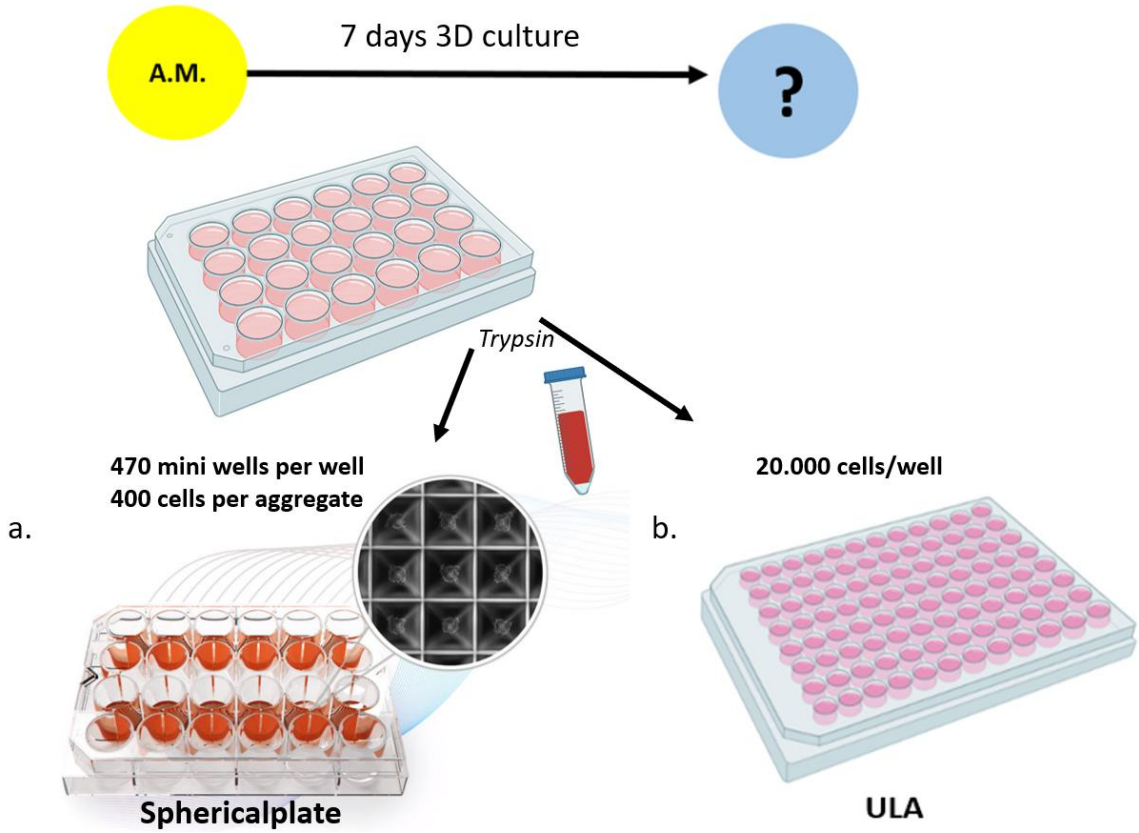


Figure.47. A.M. stage cells were trypsinized with 50% trypsin and replated in 25% M.CM final concentration of their replating medium in either sphericalplates (**a**) or ULA (**b**). Both conditions promote the interaction between the cells and their aggregation by having low attachment surfaces.

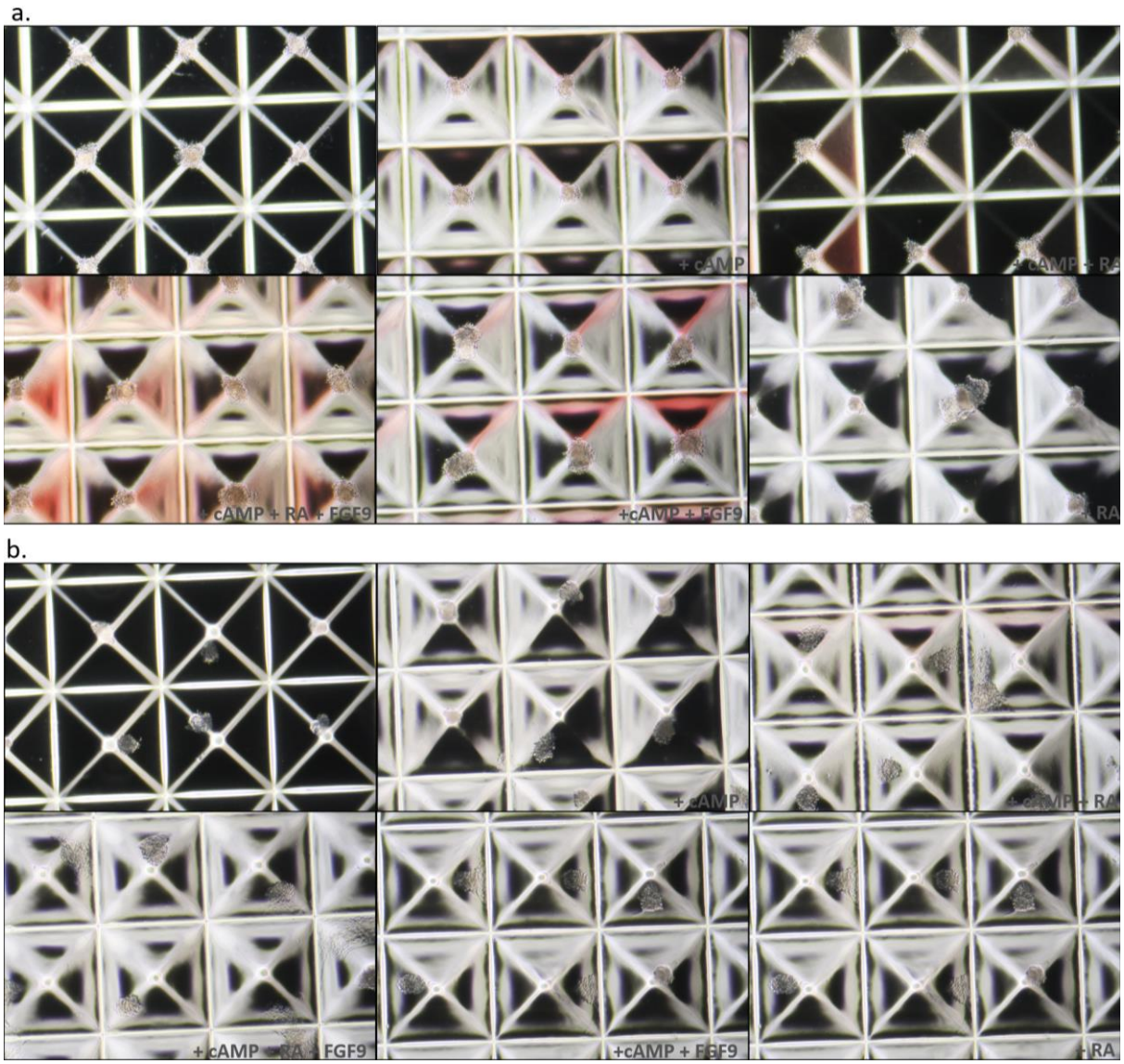


Figure.48. Experiment-1 of (Kug1) of cell growing in sphericalplates in ARPMI (**a**) and cE6 (**b**) medium. In order from left to right and top to bottom: plain medium, 8-Br-cAMP (100nM), RA (0.5uM), 8-Br-cAMP (100nM) + RA (0.5uM), 8-Br-cAMP (100nM) + RA (0.5uM) + FGF9 (100ng/ml), 8-Br-cAMP (100nM) + FGF9 (100ng/ml) and RA (0.5). The formation of the aggregates was carried out as in **Figure.47a** 10x magnification.

Sphericalplate experiments were all analysed by RT-qPCR. Samples from **Figure.46** were used as a comparison, since they were the most successful experiments including a validation of NR5A1 protein expression. We examined the expression of early steroidogenic genes (AP fate) including *Nr5a1*, *Mc2r* and *Mgarp* (**Figure.50**). *Mgarp* is a gene specifically enriched in AP fated cells on a single cells RNA atlas of E10.5 mouse, based on unpublished data from our collaborators. Remarkably, the first experiment with the Sphericalplate produced aggregates with very high *Nr5a1* expression, reaching levels half of that found in E12.5 adrenals (~10^3 compared with s.UR). The same trend could be observed in both media, showing significant upregulation of *Nr5a1* in plain media and with a further increase upon addition of 8-Br-cAMP and FGF9. By contrast, addition of RA reduced the expression of *Nr5a1* by a factor of 5 times (data not shown). Furthermore, aggregates that were grown in cE6 media had twice as much *Nr5a1* expression when compared to ARPMI media **(Figure.50a&b**). Unfortunately, these high levels of *Nr5a1* expression could not be repeated in the 2 other replicates, but the pattern

seems preserved but with lower expression of *Nr5a1*, *Mc2r* or/and *Mgarp*. In conditions with 8-Br-cAMP and FGF9 in cE6 media, Nr5a1 expression was highest, which was accompanied by five times higher expression of *Mgarp*, when compared to the rest of the samples. Also, *Mc2r* and *Mgarp* expression appeared to correlate with significant levels of *Nr5a1* expression.

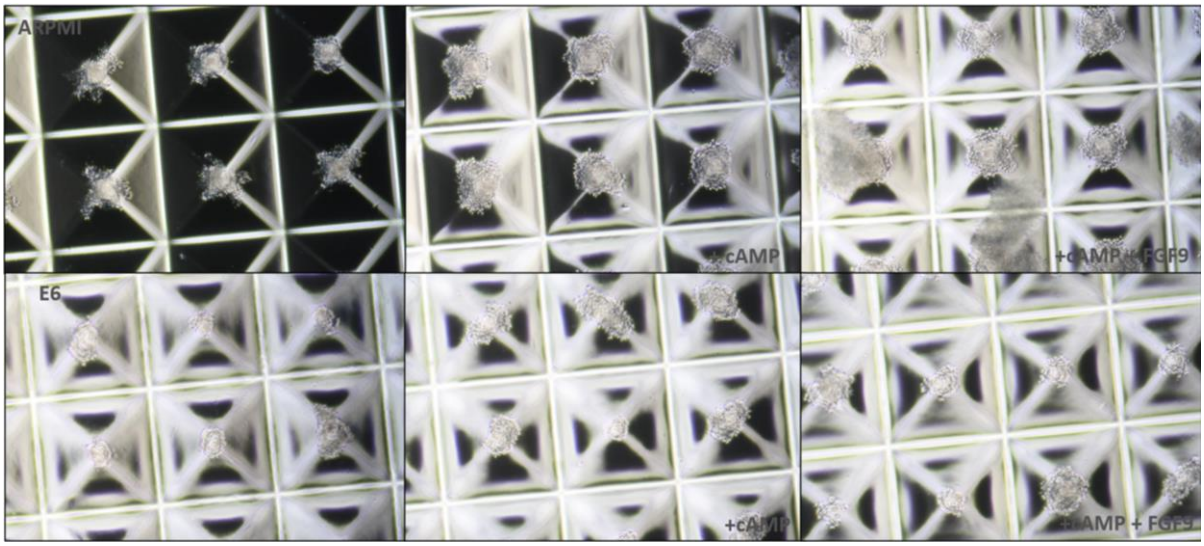


Figure.49. Experiment-3 of (Kug3) of cell growing in sphericalplates in ARPMI on top and cE6 medium at bottom. In order from left to right: plain medium, 8-Br-cAMP (100nM) and 8-Br-cAMP (100nM) + FGF9 (100ng/ml). The formation of the aggregates was carried out as in **Figure.47a.** 10x magnification.

RT-qPCR was also performed for the more general AGP markers *Wt1*, *Cited2* and *Lim9* (**Figure.50**) and for genes related with GP formation (*Foxl2, FST, Sox9*) (**Figure.51**). Both *Wt1* and *Cited2* seemed to follow the same pattern. Kug3 and 2D-ECM samples failed to express *Wt1,* but showed *Cited2* expression. *Lim9*, a gene generally considered as a gonadal maker, was only significantly expressed in Kug1 cE6/8-Br-cAMP + FGF9 and at lower levels in Kug2 ARPMI/8-Br-cAMP. For the gonadal fate, *Foxl2* and *Sox9* are considered as marker in ovarian and testis fated cells, respectively. In addition, Follistatin (*Fst*) was tested as a marker for ovarian related differentiation (Eggers et al., 2014). Although, *Fst* shows a more diffuse expression in early development, and within the pool of mesodermal cells, it has been observed at somites of E9.5 mouse embryos (de Groot et al., 2000). Thus, coupled *Fst* and *Foxl2* expression can be interpreted as an ovarian fate. Likewise, in earlier development *Sox9* has broad expression, and it can also be localised at the somites like *Fst* (Barrionuevo et al., 2006). Nevertheless, in both media and in all conditions analysed, *Sox9* expression was very low suggesting no differentiation towards the testicular lineage.

For the ovarian related genes, in general there was a co-expression of *Fst* and *Foxl2* **(Figure.52)**. On cE6 medium at the Kug1 and Kug2 experiments the expression of *Fst* was not higher than A.IM, but *Foxl2* was increasing in a pattern similar to *Nr5a1* **(Figure.50)**. This may indicate an ovarian differentiation. On Kug3 and 2D-ECM the expression of *Fst* was almost double of the other experiments, but with similar *Foxl2* levels. At the ARPMI, for Kug1 the levels of the two genes are comparable with cE6 condition, but on the Kug2 there is a more than double Foxl2 expression. More striking though is the expression levels of *Fst* on Kug3 and 2D-ECM, with *Foxl2* being much lower than expected on Kug3, suggesting not a complete ovarian fate. We can also observe a similar patterning of *Cited2* and *Fst* expression in ARPMI. An expression pattern can be observed in cE6 with *Foxl2* and

Nr5a1, which is more obvious in the samples where the *Nr5a1* expression is lower, excluding the Kug1 ones.

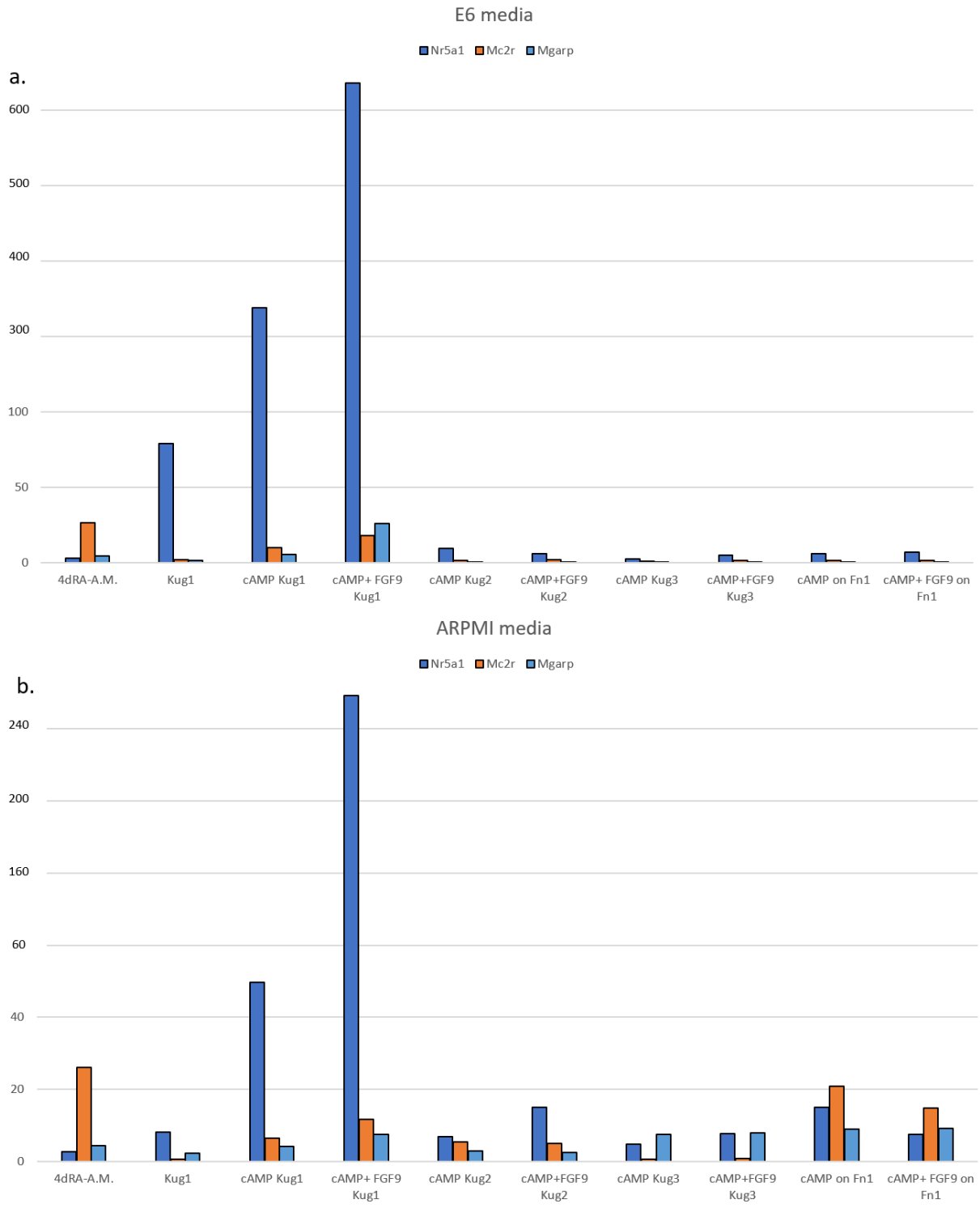


Figure.50. RT-qPCR analysis for *Nr5a1*, *Mc2r* and *Mgarp* from R1 cells generated aggregates cultured in either cE6 (**a**) or with ARPMI (**b**) media. The formation of aggregates was carried out as shown in **Figure.47b**. Kug1 samples were taken from **Figure.48,** Kug3 from **Figure.49** and cells on FN1 are samples from **Figure.46**. Kug1 are cells from plain media and the rest indicate the conditions with 8-Br-cAMP, with or without FGF9. A.M. sample was used as an input for comparison. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with *Sdha* expression.

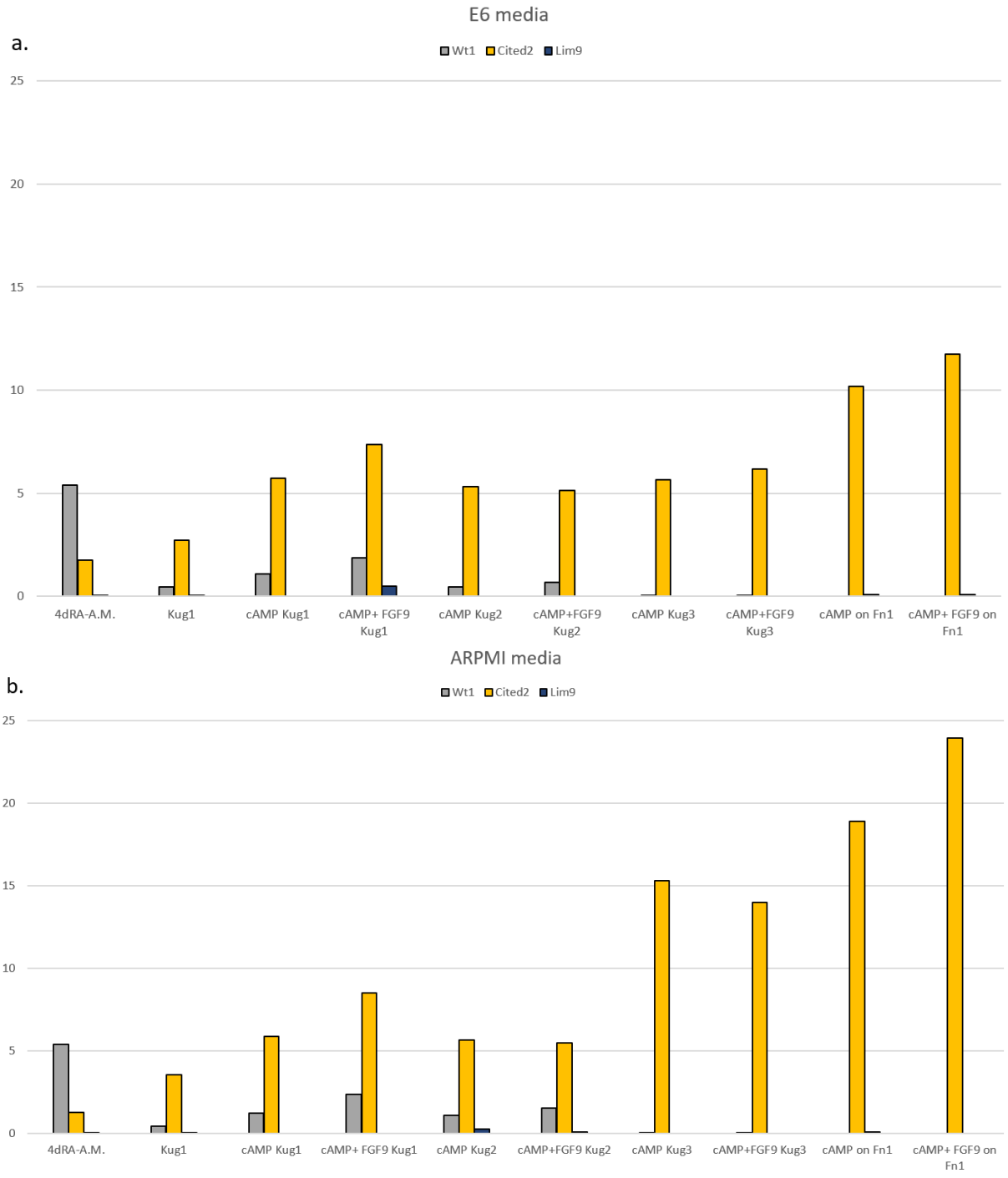


Figure.51. RT-qPCR analysis for Wt1, Cited2 and Lim9 from R1 cells generated aggregates cultured in either cE6 (**a**) or with ARPMI (**b**) medium. The formation of the aggregates was carried out as in **Figure.47b.** Kug1 samples were taken from **Figure.48,** Kug3 from **Figure.49** and cells on FN1 are samples from **Figure.46**. Kug1 are cells from plain medium and the rest indicate the conditions with 8-Br-cAMP, with or without FGF9. A.M. sample was used as an input for comparison. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with Sdha expression.

We also sought to examine a potential upregulation of genes involved in steroidogenesis and therefore analysed the expression of *Star*, *Cyp11a1*, *Cyp21a1* and *Cyp11b1*, which are involved in the cascade of steroid production in that order (**Figure.53**). In general, expression of these steroidogenic genes was higher in cultures grown in cE6 media. ARPMI does not seem to benefit the expression of these late steroidogenic markers, with the exemption of the Kug1 with 8-Br-cAMP experiments where there is a steroidogenic fate, and potentially, in a smaller degree the 2D-ECM experiment. ARPMI

media appears to favour the gonadal fate, as in that media we observed the highest expression of *Fst* and *Foxl2* during the differentiation protocol. The opposite can be said for the cE6 media, which, based on higher expression of early (**Figure.50**) and later (**Figure.53**) steroidogenic genes, appears to favour a more adrenal-like fate (although replicates did not confirm this finding). Taken together, Kug1 and to a lesser extent 2D-ECM experiments yielded cells with a steroidogenic profile resembling fetal adrenals, perhaps mixed with gonadal fated cells. Kug2 and Kug3 experiments were less successful, demonstrating the need for a highly standardized and tightly controlled experiments. In conclusion, the cells and the protocol have the potential to generate AP cells, but further fine-tuning is needed to restrict further the differentiation.

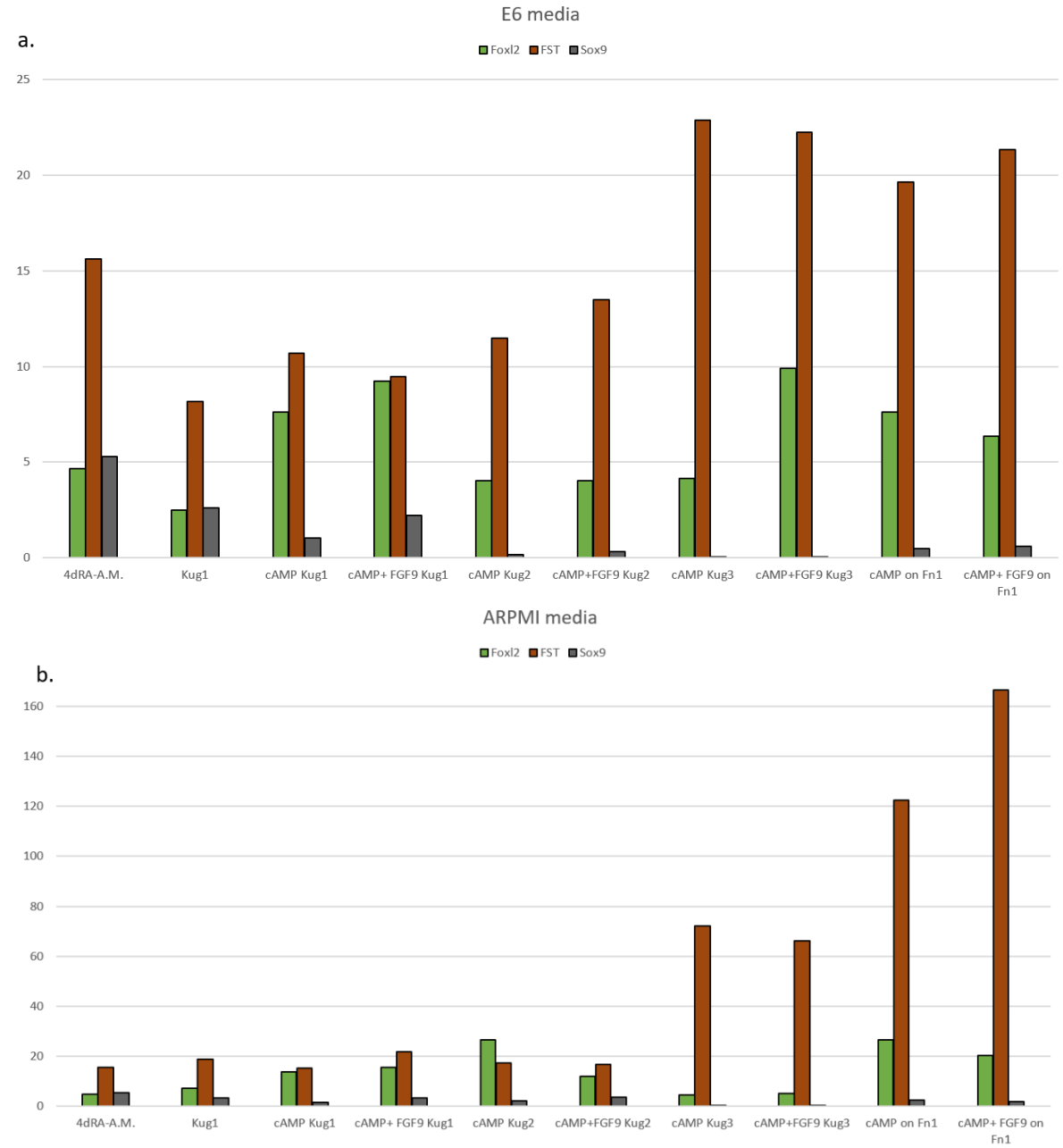


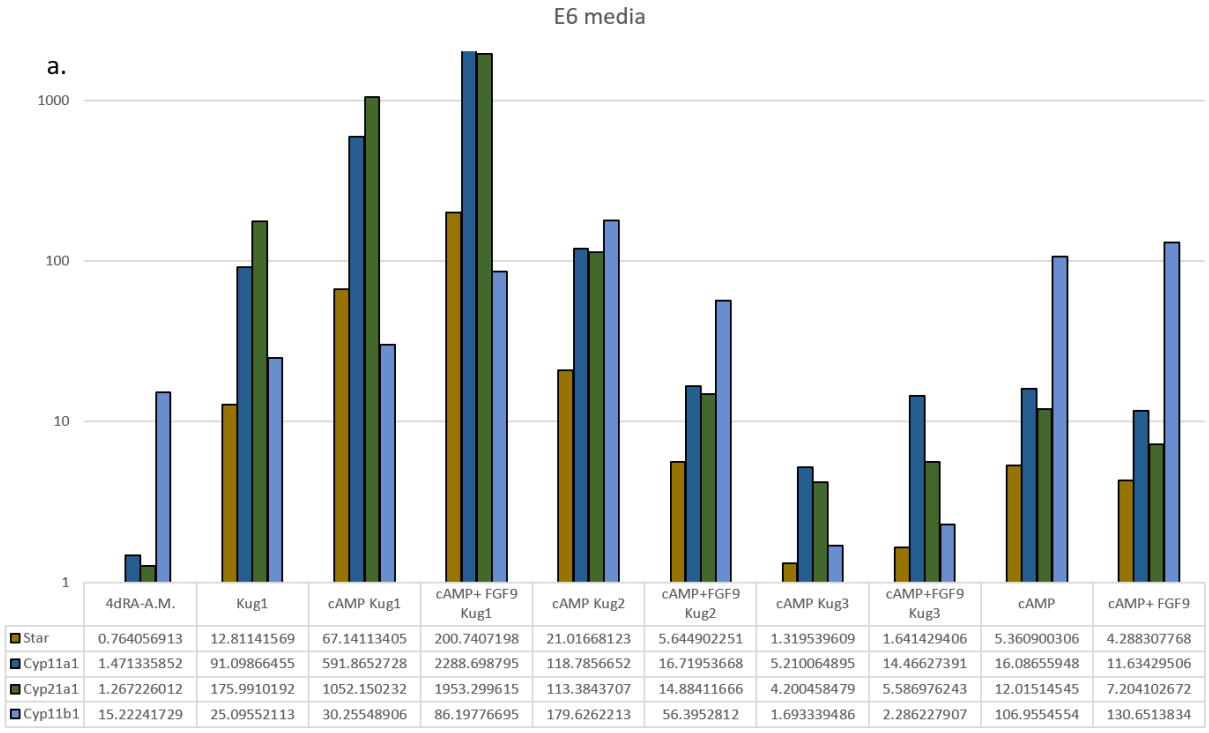
Figure.52. RT-qPCR analysis for Foxl2, FST and Sox9 from R1 cells generated aggregates cultured in either cE6 (**a**) or with ARPMI (**b**) medium. The formation of the aggregates was carried out as in **Figure.47b.** Kug1 samples were taken from **Figure.48,** Kug3 from **Figure.49** and cells on FN1 are samples from **Figure.46**. Kug1 are cells from plain medium and the rest indicate the conditions with 8-Br-cAMP, with or without FGF9. A.M. sample was used as an input for comparison. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with Sdha expression.

2.4.b. Bl6 cells.

In parallel to the R1 cells we also performed the same experiment on Sphericalplate with the *Mc2r-Gfp* cell line. Although these cells are not differentiating properly due to high cell death during the protocol, we wanted to see if the 3D niche will be beneficial. Moreover, we reasoned that 3D conditions may allow strong expression of *Mc2r* and thus the detection of the GFP protein via live imaging. The same protocol was followed as in **Figure.48** and the development of the organoids was followed with live imaging using MuviCyte; green and red channels were used for *Mc2r* and unspecific/dead cells tracking respectively. Unfortunately, cells failed to thrive in these conditions and the experiment had to be stopped after 4 days. However, GFP-positive cells were observed in low numbers in conditions with cE6 8-Br-cAMP with or without FGF9 and in ARPMI with 8-Br-cAMP conditions (**Figure.54**). Some aggregates were spotted to have strong GFP signal in ARPMI/8-Br-cAMP and cE6/8-Br-cAMP, while the cE6 8-Br-cAMP with FGF9 condition showed in general weaker signals. Since the cells were dying proper isolation of RNA for further analysis was not possible. These preliminary experiments are encouraging, but additional analysis on the molecular level (qPCR) and further refinement will need to be performed in future experiments.

In conjunction, we aimed to replicate the experiment from **Figure.48** in ULA plates (**Figure.47b**). The benefits of moving to ULA plates are the higher reproducibility of differentiation, since there is one aggregate per well. To determine the best seeding concentration, R1 cells were plated at a concentration of 500, 2.000, 5.000 and 20.000 cells/well. Six aggregates were pooled and analysed per condition via RT-qPCR. The conditions with 500 and 2.000 cells per aggregate did not yield any RNA after extraction, possibly due to a low number of cells. The condition of 20.000 cells per aggregate showed the highest level of *Nr5a1* (10-fold higher than with A.M.) coupled with *Mc2r* expression and was chosen for further experiments. For further investigation we wanted to test Bl6 cells and thus validate similar results for two different mESCs lines. Feeder-less cells were used for this experiment, thus necessitating minor adjustments regarding the maintenance of mESCs in culture, but not in the differentiation protocol up to the A.M. stage. Also, in this experiment, we added FN1 during aggregation after the cells were disassociated from A.M. stage. The aim of this was to see if the cells could incorporate the ECM protein in their aggregate niche, and if this was beneficial for the AGP-AP fating of them.

Using this setup, we were able to replicate to a certain degree the results we obtained from the Kug1 experiment and to follow up the general expression patterns of the genes from the Kug1-3 experiments. *Mgarp* showed no significant expression in any of the conditions analysed. *Mc2r* had higher expression in cE6 conditions, while the addition of FN1 did not interfere with its expression. On the other hand, *Nr5a1* was generally higher in cE6 conditions, and while the addition of FN1 reduced its expression on cE6 8-Br-cAMP+FGF9, it was also increased in the conditions with 8-Br-cAMP alone (both in cE6 and ARPMI) (**Figure.55**). *Cited2* and *Wt1* did not vary dramatically between the conditions, and *Lim9* was not expressed (**Figure.56**). After examining the early gonadal markers *Foxl2*, *Fst* and *Sox9* in this experimental setup, we saw a higher expression of female related genes (*Foxl2*, *Fst*) when the aggregates were formed and cultured in ARPMI (**Figure.57**). *Sox9* expression was not significantly expressed in any conditions analysed. A reduction of *Foxl2* expression was noted in the conditions were FN1 was present, especially in cE6 condition. Similarly, *Fst* was reduced in the presence of FN1 during aggregate formation in cE6 media, while in ARPMI the presence of FN1 did not lead to significant changes.



	4dRA-A.M.	Kug1	cAMP Kug1	cAMP+ FGF9 Kug1	cAMP Kug2	cAMP+FGF9 Kug2	cAMP Kug3	cAMP+FGF9 Kug3	cAMP	cAMP+ FGF9
Star	0.764056913	12.81141569	67.14113405	200.7407198	21.01668123	5.644902251	1.319539609	1.641429406	5.360900306	4.288307768
Cyp11a1	1.471335852	91.09866455	591.8652728	2288.698795	118.7856652	16.71953668	5.210064895	14.46627391	16.08655948	11.63429506
Cyp21a1	1.267226012	175.9910192	1052.150232	1953.299615	113.3843707	14.88411666	4.200458479	5.586976243	12.01514545	7.204102672
Cyp11b1	15.22241729	25.09552113	30.25548906	86.19776695	179.6262213	56.3952812	1.693339486	2.286227907	106.9554554	130.6513834

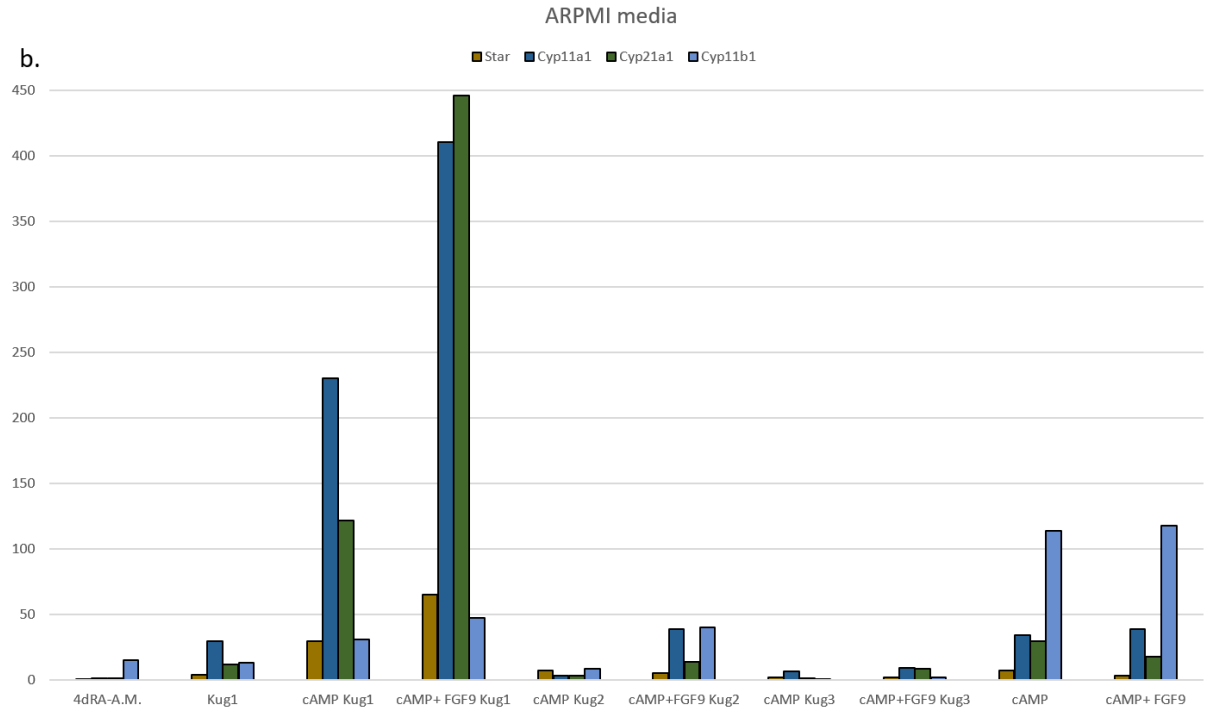


Figure.53. RT-qPCR analysis for *Star*, *Cyp11a1*, *Cyp21a1* and *Cyp11b1* from R1 cells generated aggregates, cultured in either cE6 (**a**) or with ARPMI (**b**) media. The formation of aggregates was carried out as in **Figure.47b**. Kug1 samples were taken from **Figure.48,** Kug3 from **Figure.49** and cells on FN1 are samples from **Figure.46**. Kug1 are cells from plain media and the rest indicate the conditions with 8-Br-cAMP, with or without FGF9. A.M. sample was used as an input for comparison. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with *Sdha* expression.

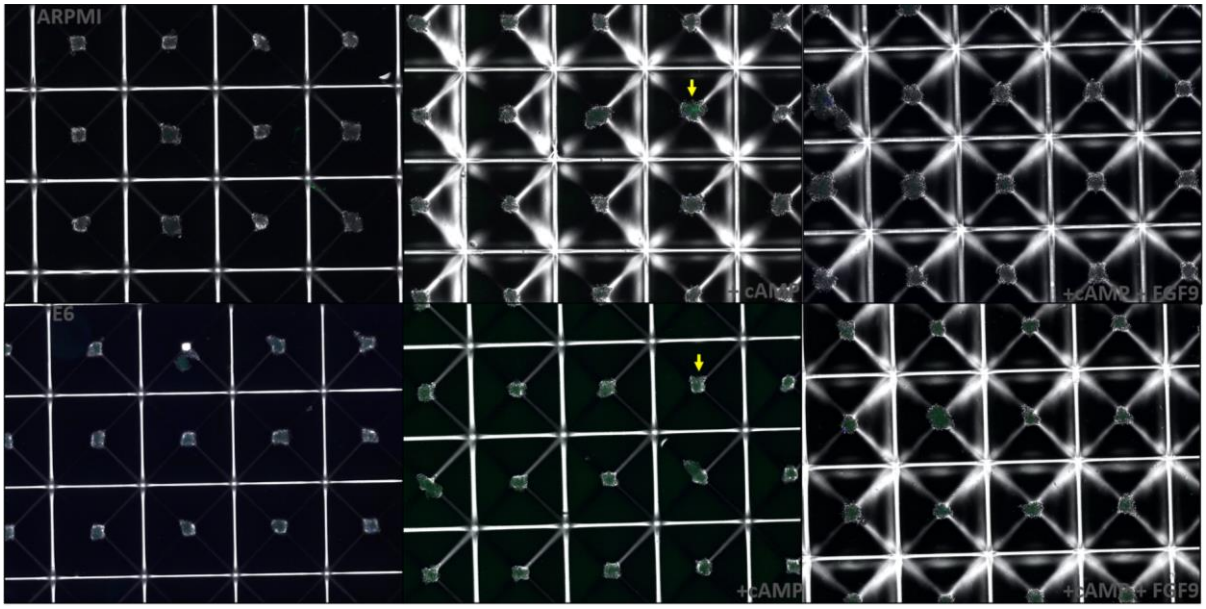


Figure.54. Experiment-4 of (Kug4) of Mc2r-Gfp cell growing in sphericalplates in ARPMI on top and cE6 media at bottom. In order from left to right: plain media, 8-Br-cAMP (100nM) and 8-Br-cAMP (100nM) + FGF9 (100ng/ml). The formation of the aggregates was carried out as in **Figure.47a**. The GFP (green colour) has been fused with the brightfield and red channel (in blue colour) is used to detect background signal. Yellow arrows indicate strong GFP expression.4x magnification from MuviCyte instrument.

Once again, we questioned the later steroidogenic potential of these aggregates by examining the same markers as in R1 cells experiment (**Figure.53**). No significant expression of *Cyp11b1* was detected, and the remainder of the markers was reduced in the presence of FN1 in both media (**Figure.58**). The expression levels of *Star* were higher in the conditions containing cE6 media, and the addition of FGF9 had negative effect on its maintenance. *Cyp11a1* had a more interesting pattern, where in the absence of FN1, FGF9 repressed the expression of it only in ARPMI media, while in cE6 it had higher expression when 8-Br-cAMP was added. Also, when the aggregates were formed with the presence of FN1, *Cyp11a1* was only significantly expressed with the addition of 8-Br-cAMP; an effect which was increased when cells were cultured in cE6 media and even more when combined with FGF9 addition. Finally, *Cyp21a1* was only expressed with the addition of FGF9 in ARPMI media, when FN1 was added during the formation of the aggregates.

In summary, this experiment suggests that differentiation of Bl6 cells is delayed when compared with R1 cells, as evidenced by both early (**Figure.50,55**) and late (**Figure.53,58**) steroidogenic markers. Our previous observation that cE6 and 8-Br-cAMP favour the AP fate seems to be partially confirmed by the high levels of *Nr5a1* expression, followed by *Mc2r*, *Star* and *Cyp11a1* expression. Interestingly, the cells had a greater tendency to differentiate along the gonadal fate, especially when cultured in ARPMI media, something which could be at least partially supressed in cE6 media in the presence of FN1. It is possible that the aggregates needed more time in culture, or additional differentiation cues were present in the R1 cells experiment. These results represent a partial replication of the Sphericalplate protocol with a different cell line. Once again further optimization is needed.

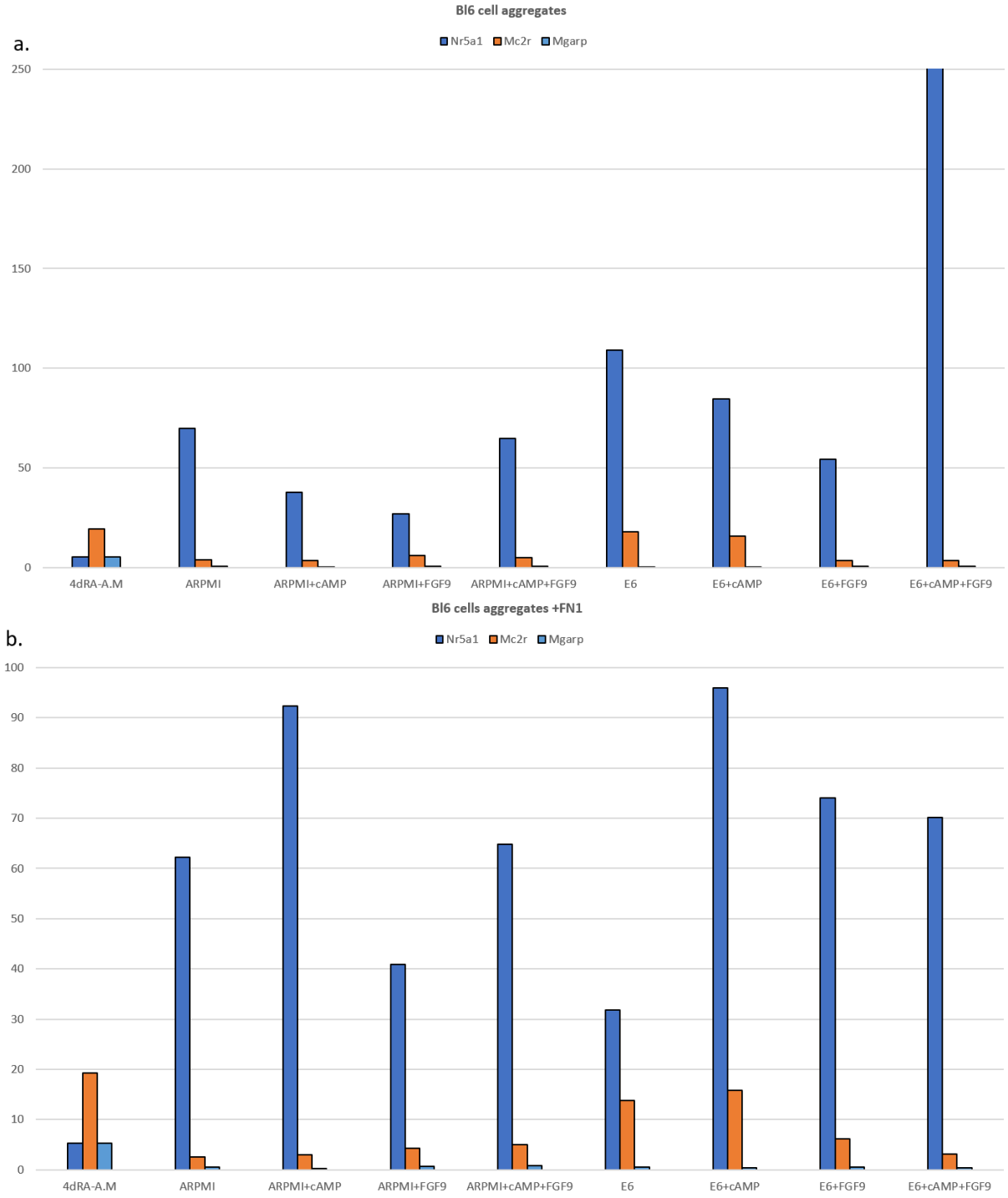


Figure.55. RT-qPCR analysis for *Nr5a1*, *Mc2r*, *Mgarp* and *Star* from Bl6 generated aggregates cultured in either ARPMI or cE6 media, without (**a**) or with FN1 (**b**) addition during the formation. The formation of aggregates was carried out as in **Figure.47b**. A.M. sample was used as an input for comparison. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with *Sdha* expression.

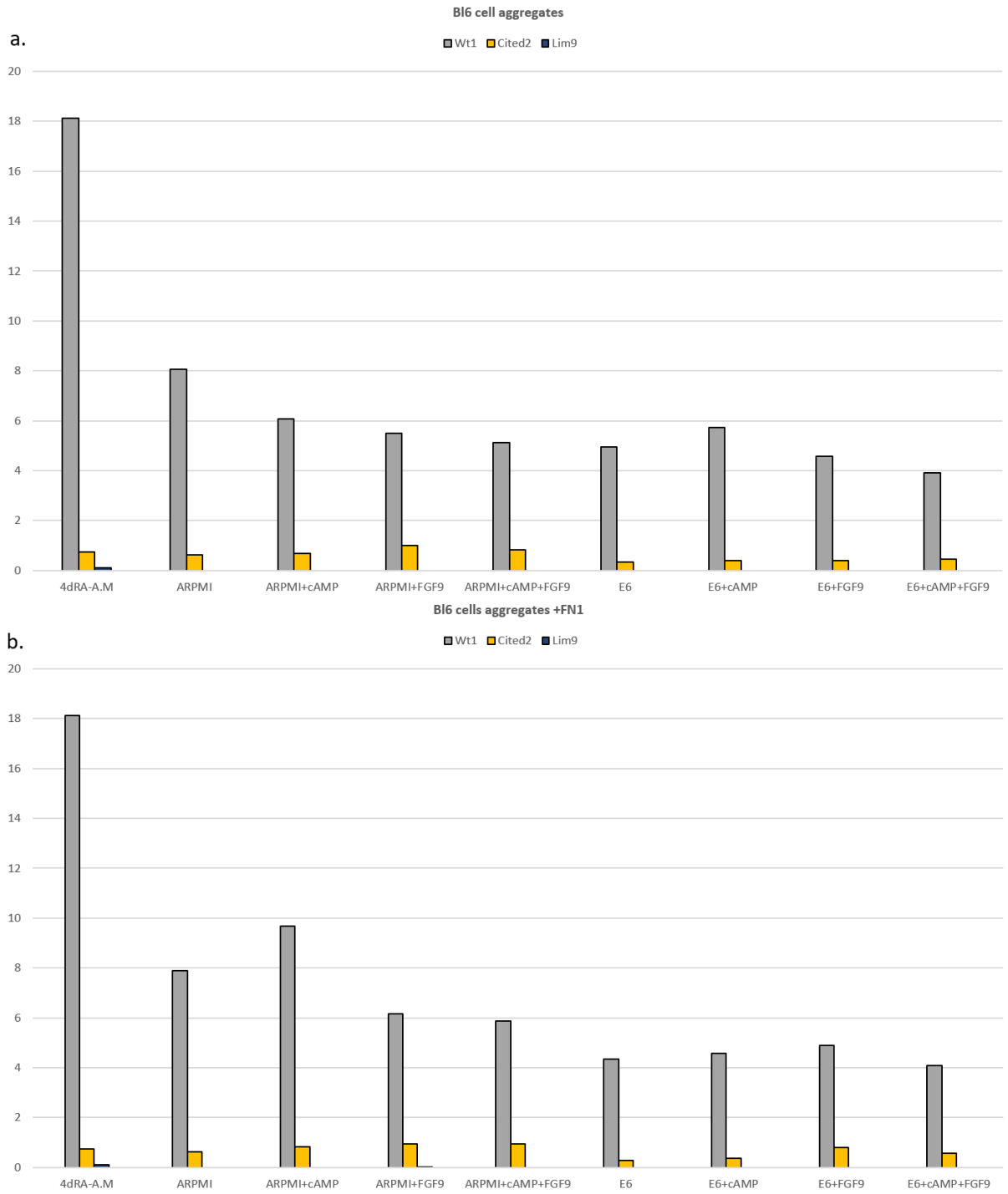


Figure.56. RT-qPCR analysis for *Wt1*, *Cited2* and *Lim9* from Bl6 generated aggregates cultured in either ARPMI or cE6 media, without (**a**) or with FN1 (**b**) addition during the formation. The formation of aggregates was carried out as in **Figure.47b**. A.M. sample was used as an input for comparison. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with *Sdha* expression.

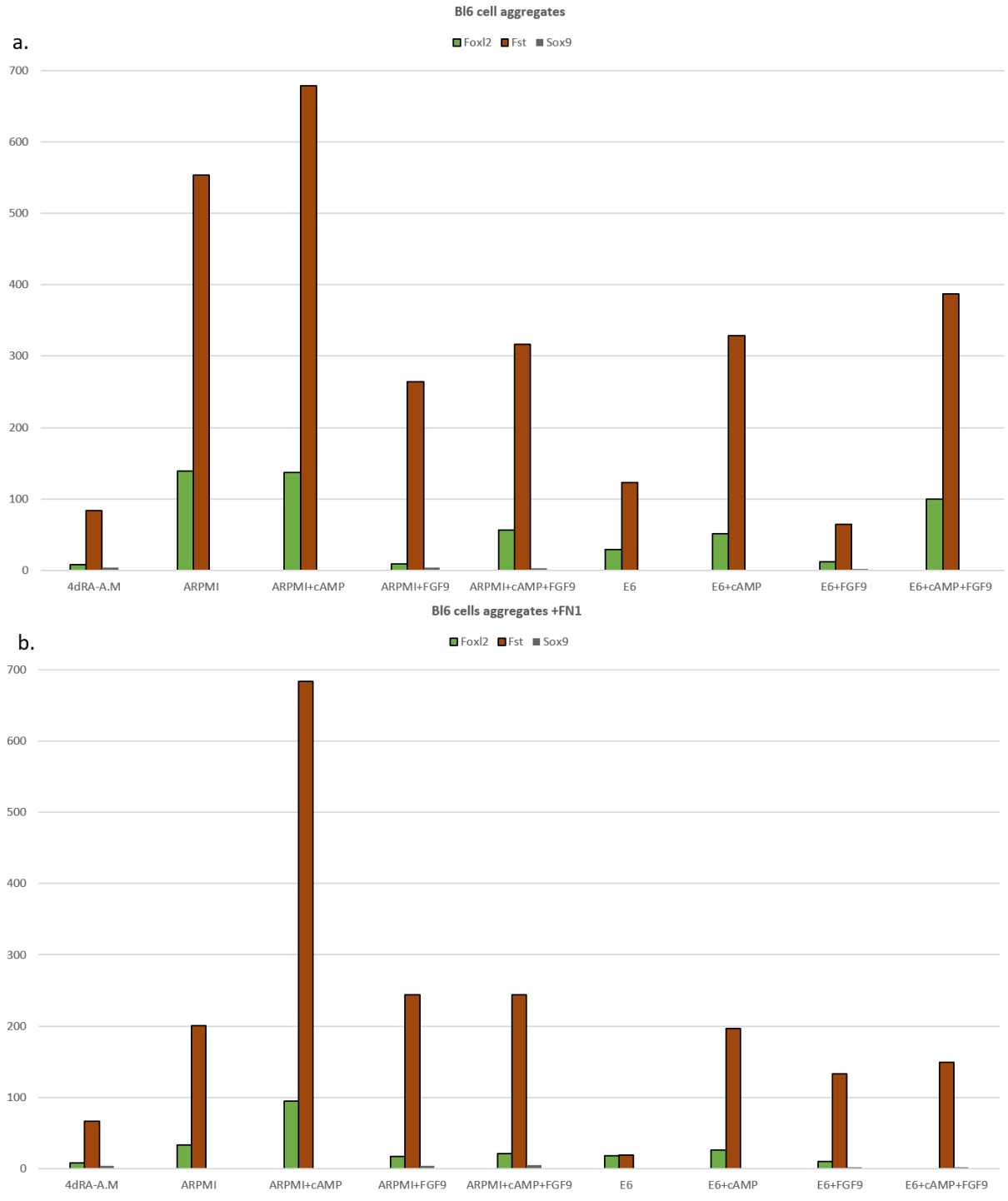


Figure.57. RT-qPCR analysis *Foxl2* and *Fst* from Bl6 generated aggregates cultured in either ARPMI or cE6 media, without (**a**) or with FN1 (**b**) addition during the formation. The formation of aggregates was carried out as in **Figure.47b.** A.M. sample was used as an input for comparison. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with *Sdha* expression.

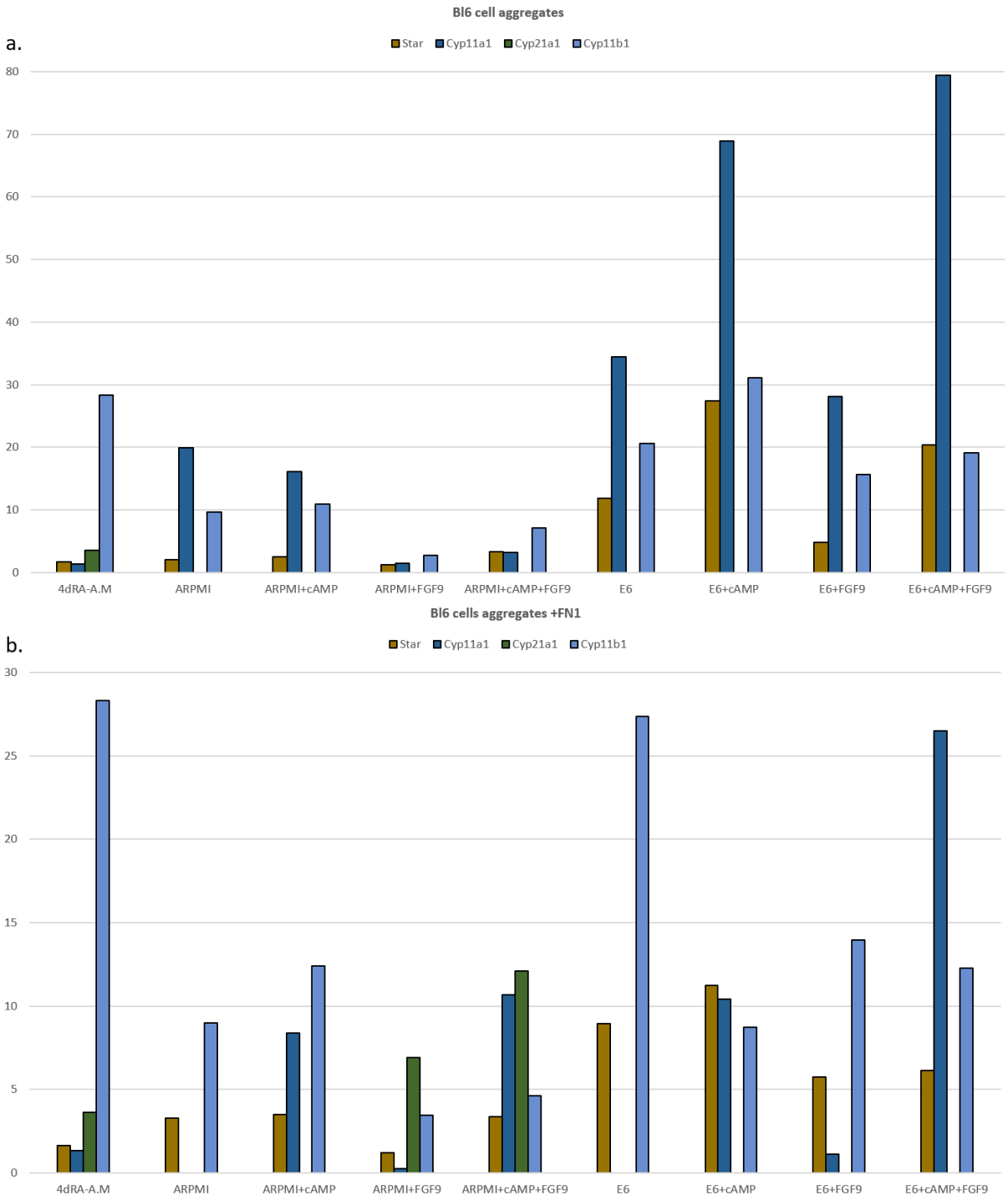


Figure.58. RT-qPCR analysis for *Star*, *Cyp11a1*, *Cyp21a1* and *Cyp11b1* from Bl6 generated aggregates cultured in either ARPMI or cE6 media, without (**a**) or with FN1 (**b**) addition during their formation. The formation of the aggregates was carried out as in **Figure.47b.** A.M. sample was used as an input for comparison. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with *Sdha* expression.

2.4.c. Additional aggregate experiments.

Taking in consideration the experiments done in R1 and Bl6 cells, cE6 and ARPMI media, and in ULA and sphericalplates, we sought out to minimize and define further the conditions. In the protocol used in this thesis, R1 cells are more fitted for AP differentiation, thus further experimentation was not done with the Bl6. Although the cE6 medium has showed good results, it can have great batch to batch differences because of it is homemade status, and it can be potentially more difficult to be tested by other researchers or teams; thus, E6 commercial media (TeSR™-E6) was used for the next experiments. We still treated half of the samples with ARPMI medium as a reference point of the differentiation protocol, and we did experiments in both ULA (4) and sphericalplates (2). Interestingly, in both ways of aggregate formation in the presence of 8-Br-cAMP and in E6 medium particularly, the cells showcased a steroidogenic profile by expressing high levels of *Nr5a1*, *Star*, *Cyp11a1* and *Cyp21a1* (**Figure.59**). While analysing the Sphericalplate experiment we used also the 8-Br-cAMP treated Kug1 samples (**Figure.59a**) for the RT-qPCR analysis, which was also the sample with the highest expression profiles in this graph. Remarkably, the Sphericalplate samples had expression levels comparable with the mouse adrenal of E14.5, when the aggregates grew in E6 medium.

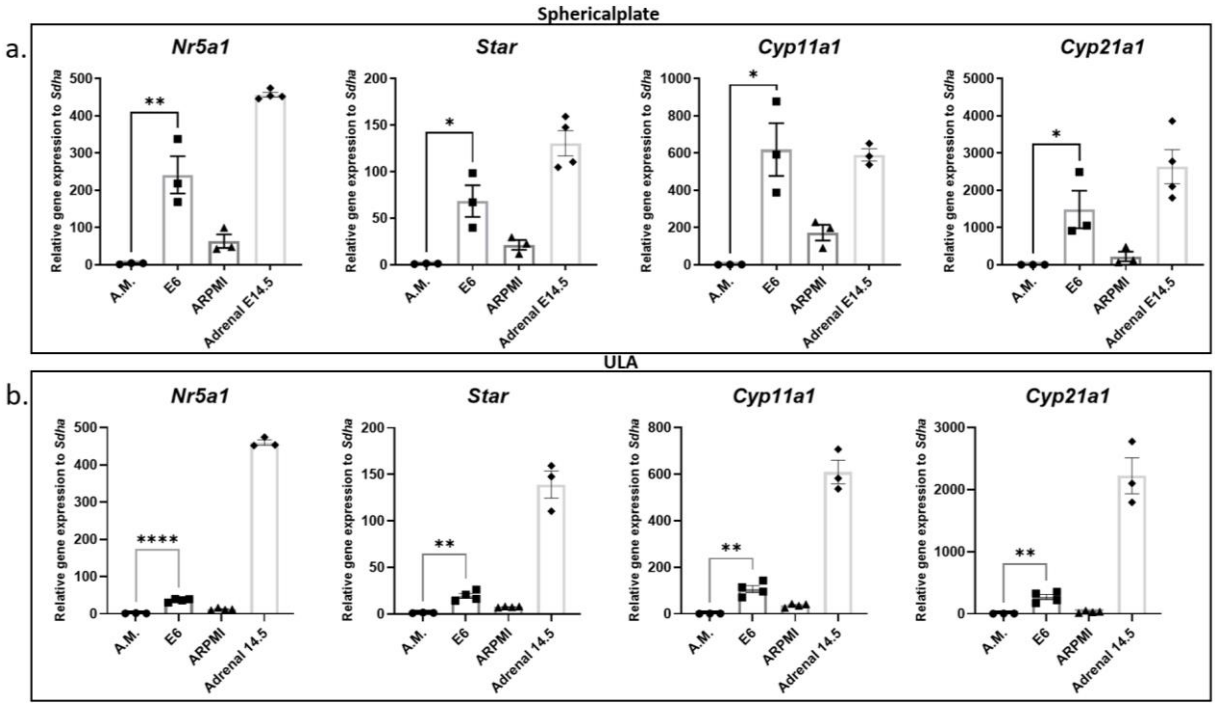


Figure.59. RT-qPCR analysis for *Nr5a1*, *Star*, *Cyp11a1* and *Cyp21a1* from R1 generated aggregates cultured in either ARPMI or E6 media, made in (**a**) Sphericalplates or (**b**) ULA plates and treated with 8-Br-cAMP. For (**a**) we used also the Kug1 sample for E6 and ARPMI. The formation of the aggregates was carried out as in **Figure.47**. A.M. and adrenal at E14.5 samples were used as an input for comparison. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with *Sdha* expression.

From this series of experiments, we can observe with ease the superiority of the E6 media over ARPMI in this *in vitro* differentiation protocol for steroidogenic cells. We can also observe the higher expression levels of these genes when aggregates are formed and treated in sphericalplates over the ULA plates. This difference may be resulted from the sphericalplates producing smaller aggregates, something which we saw that impacted also the differentiation of R1 and Bl6 cells in ULA plates at the previous experiments. Additionally, while in each well of the ULA there is a single aggregate, in the sphericalplates there are around 450 aggregates, and they may be communicating with each other via their secretome. These aggregates secrete factors in the medium, where they can reach to each other,

and they may be promoting the differentiation or maintain the steroidogenesis between them. Both of these reasons may be responsible for the better results of the sphericalplates, but further validation is needed.

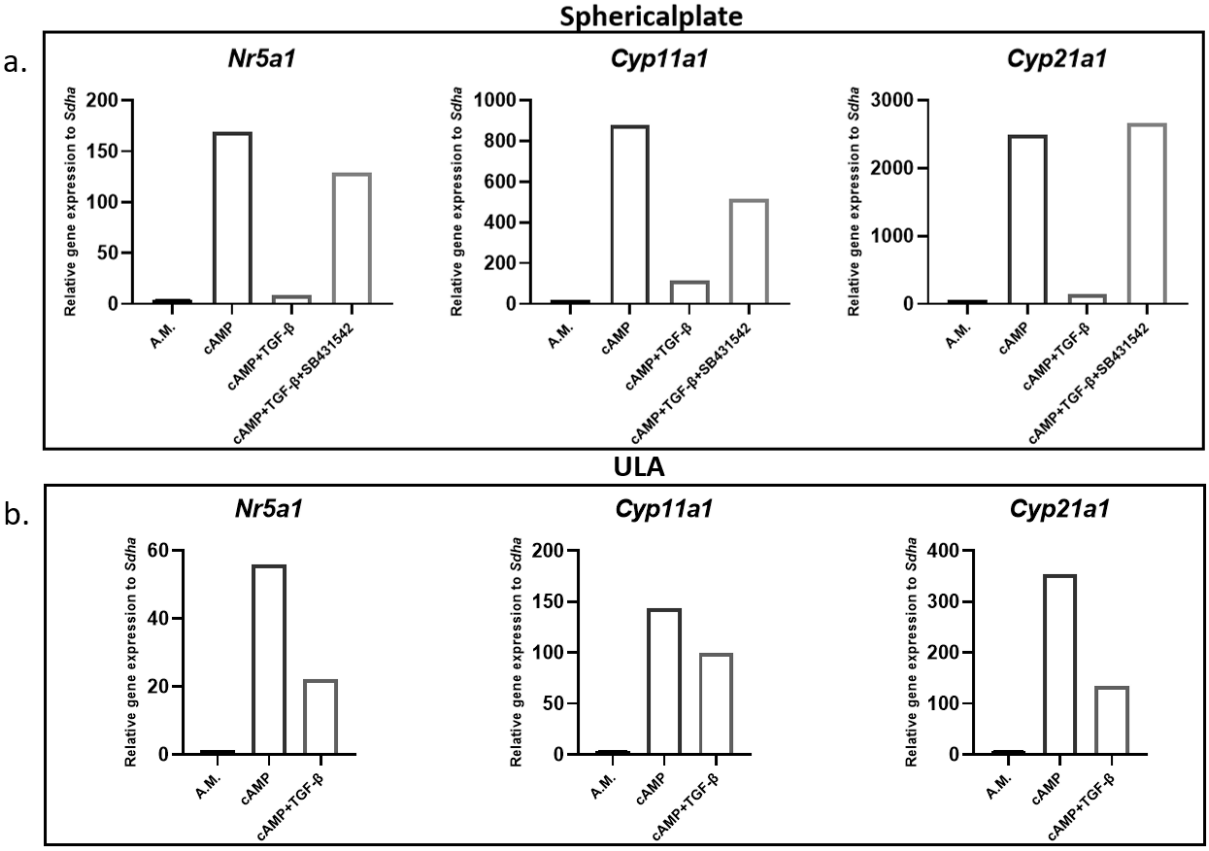


Figure.60. RT-qPCR analysis for *Nr5a1*, *Cyp11a1* and *Cyp21a1* from R1 generated aggregates cultured in E6 media, made in (**a**) Sphericalplates or (**b**) ULA plates and treated with either 8-Br-cAMP, 8-Br-cAMP + TGF-β or 8-Br-cAMP + TGF-β + SB431542 (TGF-β inhibitor). The formation of the aggregates was carried out as in **Figure.47.** A.M. samples was used as an input for comparison. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with *Sdha* expression.

In the next experiment we wanted to address the functionality of these aggregates, basically if they respond to exogenous signals. We wanted to validate that the steroidogenic profile of these aggregates is active, and that it is not an artifact of the *in vitro* system. Indeed, after treating the cells at the fourth day of aggregate formation in E6 media with Transforming growth factor-beta (TGF-β), an inhibitor of steroidogenesis (Attia et al., 2000; Gupta et al., 1992; Hotta & Baird, 1986; Lehmann et al., 2005; Stelcer et al., 2020a), the expression levels of the steroidogenic genes fell when compared with the condition of 8-Br-cAMP treatment alone (**Figure.60**). The effect of the inhibition was more prevalent in the sphericalplate experiment (**Figure.60a**) with having more than 10 times less expression, compare to the ULA one where the expression levels were halved (**Figure.60b**). This effect was largely inhibited when SB431542, a TGF-β inhibitor, was added in the mix of factors at the sphericalplate experiment (**Figure.60a**), while it did not have any significant result on the ULA plates (data not shown). With this experiment we can see the dynamism of the system and the responsiveness of the cells, especially in the sphericalplates. Although these results are reassuring, from only this experiment we cannot know if we indeed inhibited the steroidogenic profile of active steroidogenic cells, or we interfered with a key point of their differentiation.

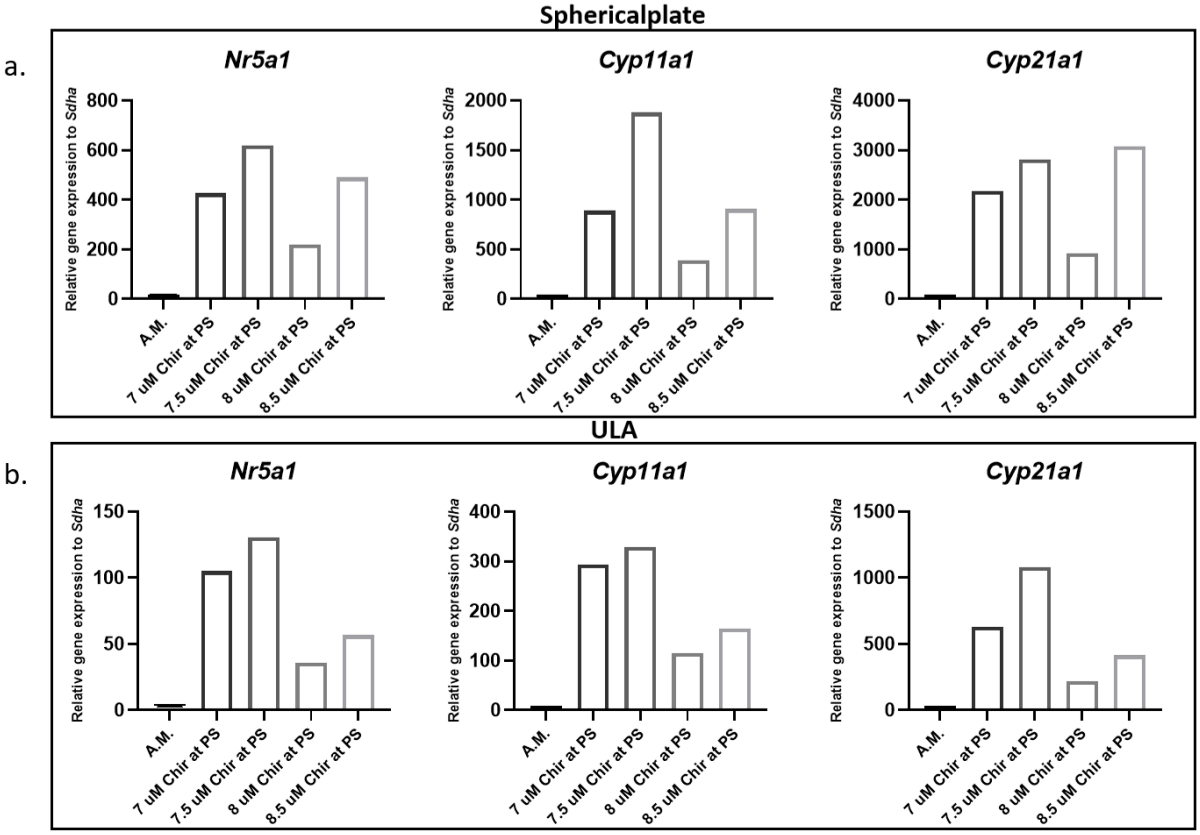


Figure.61. RT-qPCR analysis for *Nr5a1*, *Cyp11a1* and *Cyp21a1* from R1 generated aggregates cultured in E6 media made in (**a**) Sphericalplates or (**b**) ULA plates and treated with 8-Br-cAMP. The aggregates were formed from differentiated cells following the **Figure.11a,** but with treated either 7, 7.5, 8 or 8.5 uM of Chir at the PS state. The formation of the aggregates was carried out as in **Figure.47.** A.M. samples was used as an input for comparison. All the results were compared with the s.UR region of E9.5 mouse (**Figure.21c**) and normalised with *Sdha* expression.

Finally, we wondered if we could improve the steroidogenic profile/differentiation of the cells by making adjudgments to earlier steps of the protocol. As we have mentioned already, the formation of the PS is the first of the pivotal differentiation points of this *in vitro* protocol. We repeated the protocol of differentiation as before, but this time we target the Chir concentration used for the formation of the PS over the duration of 2 days. We tested 7, 7.5 and 8.5 uM of the Chir, and we also used the reference concentration of 8 uM, which we have used in all the previous experiments. Aggregates were formed from the four different conditions of PS formation in both sphericalplates and ULA plates in E6 media, as mentioned before, and treated with 8-Br-cAMP thereafter (**Figure.61**). In our surprise, the reference condition had the lowest expression levels of steroidogenic genes in both sphericalplates (**Figure.61a**) and ULA plates (**Figure.61b**), while the rest conditions showcased same gene expression tendencies in both plates. The steroidogenic profile of the aggregates is stronger in the lower concentration of Chir, with 7.5 uM being the best one.

To further validate these findings, we sent the already gathered endpoint media of **Figure.61a** for the 7 and 7.5 uM of Chir in PS conditions for steroidomics analysis. Together with these two samples we tested two other samples as negative controls. Firstly, we tested M.CM gathered from previous step of the differentiation and used for this differentiation, and the MEF CM which was also used for the EpiSC differentiation (**Figure.62**). The steroidomics analysis follows the trend of the qPCR analysis, where the 7.5 uM Chir condition has stronger steroidogenic profile than the 7 uM one (**Figure.61**). We can clearly see that the media of the aggregates contains small concentrations of specific steroid

hormones, while the control samples have non-detectable or close to zero concentration of the same steroid hormones. More particularly, as seen in **Figure.A1**, we detected the predecessors of Corticosterone and Aldosterone, 11-Deoxycorticosterone and 11-Deoxycortisol respectively, but only Corticosterone as the final product of the steroidogenesis. Furthermore, the inactive form of corticosterone, 11-Dehydrocorticosterone (BURTON et al., 1953; M. C. Holmes et al., 2001; SPRAGUE et al., 1950), was also detected in relatively high levels. Interestingly, we were able to detect small amounts of Androstenedione. This hormone is made in the adrenal glands as well as the testes and ovaries, which serves as a precursor of testosterone and estrone in males and females (Horton & Tait, 1966; Kley et al., 1980; MACDONALD et al., 1967; Perel et al., 1980).

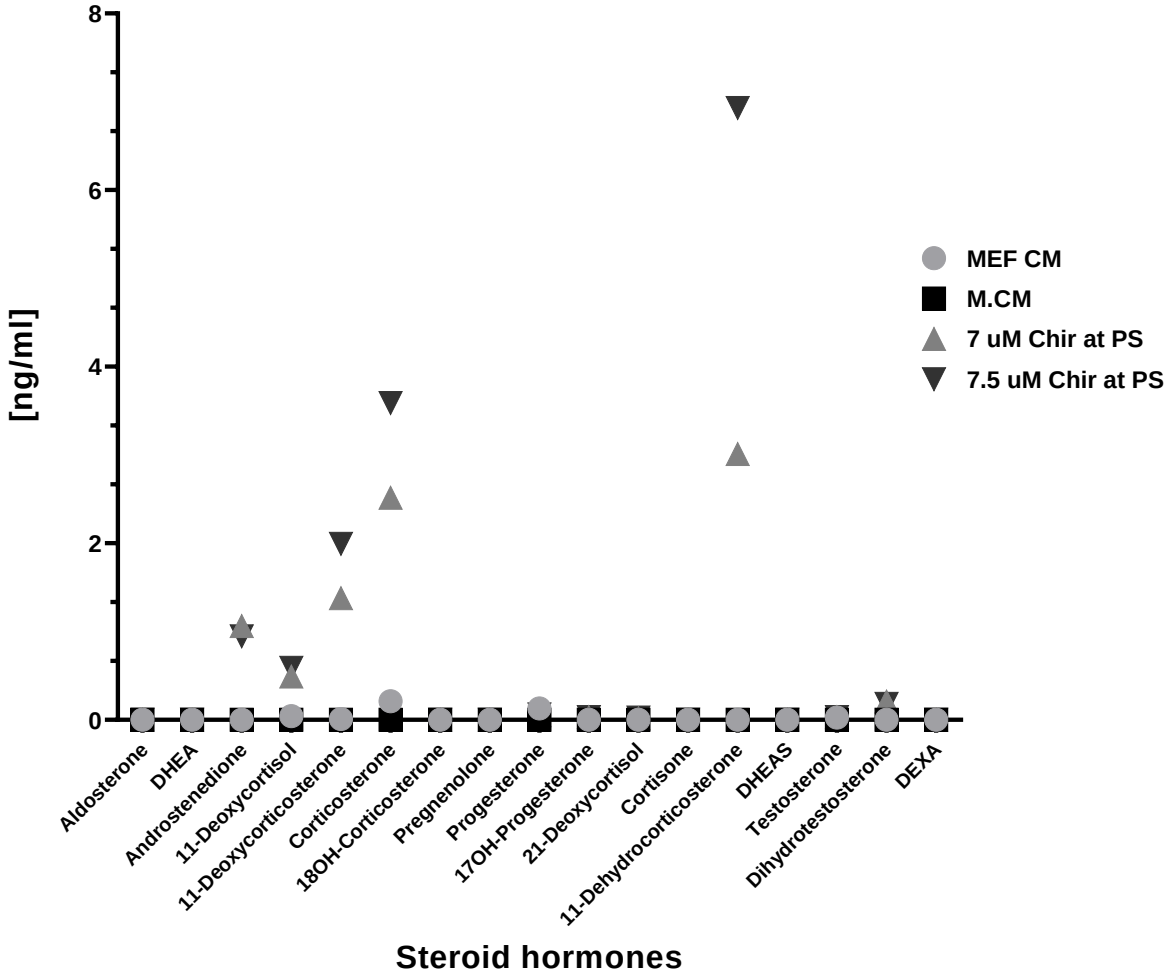


Figure.62. Steroidomics analysis for media sample from **Figure.61**, after the 7 days of aggregate formation. The conditions tested are those of 7 and 7.5 uM of Chir during PS formation. Media sample from MEF CM and M.CM, which was used for the differentiation, were used as negative controls.

From the steroidomics results we can observe that the main active steroidogenic profile of the cells leads to the path of Glucocorticoids production, as seen by the relatively high detection levels of 11-Deoxycorticosterone, 11-Dehydrocorticosterone and Corticosterone. Another critical observation is the fact that there is no detection of earlier products of steroidogenesis like Pregnenolone or Progesterone. This outcome may indicate that the steroidogenesis is at an endpoint and further signalling is required for the cells to re-enter a full steroidogenesis, or there is full conversion toward the end-product of steroidogenesis. Regardless the reason, more experiments have to be done in order to validate this protocol and both of these observations have to be taken in consideration before moving forward.

2.5. In vitro differentiation of hIPSCs.

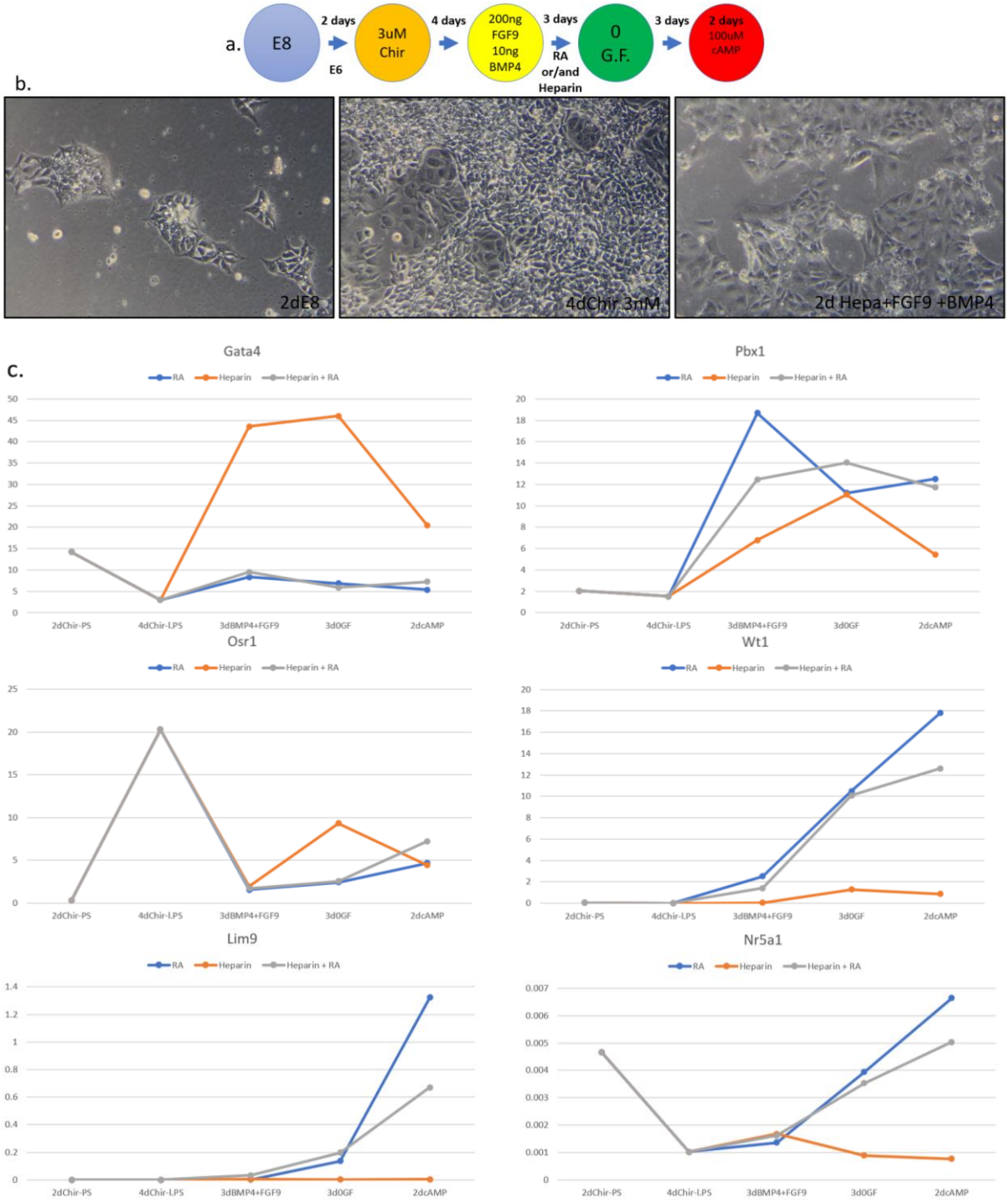
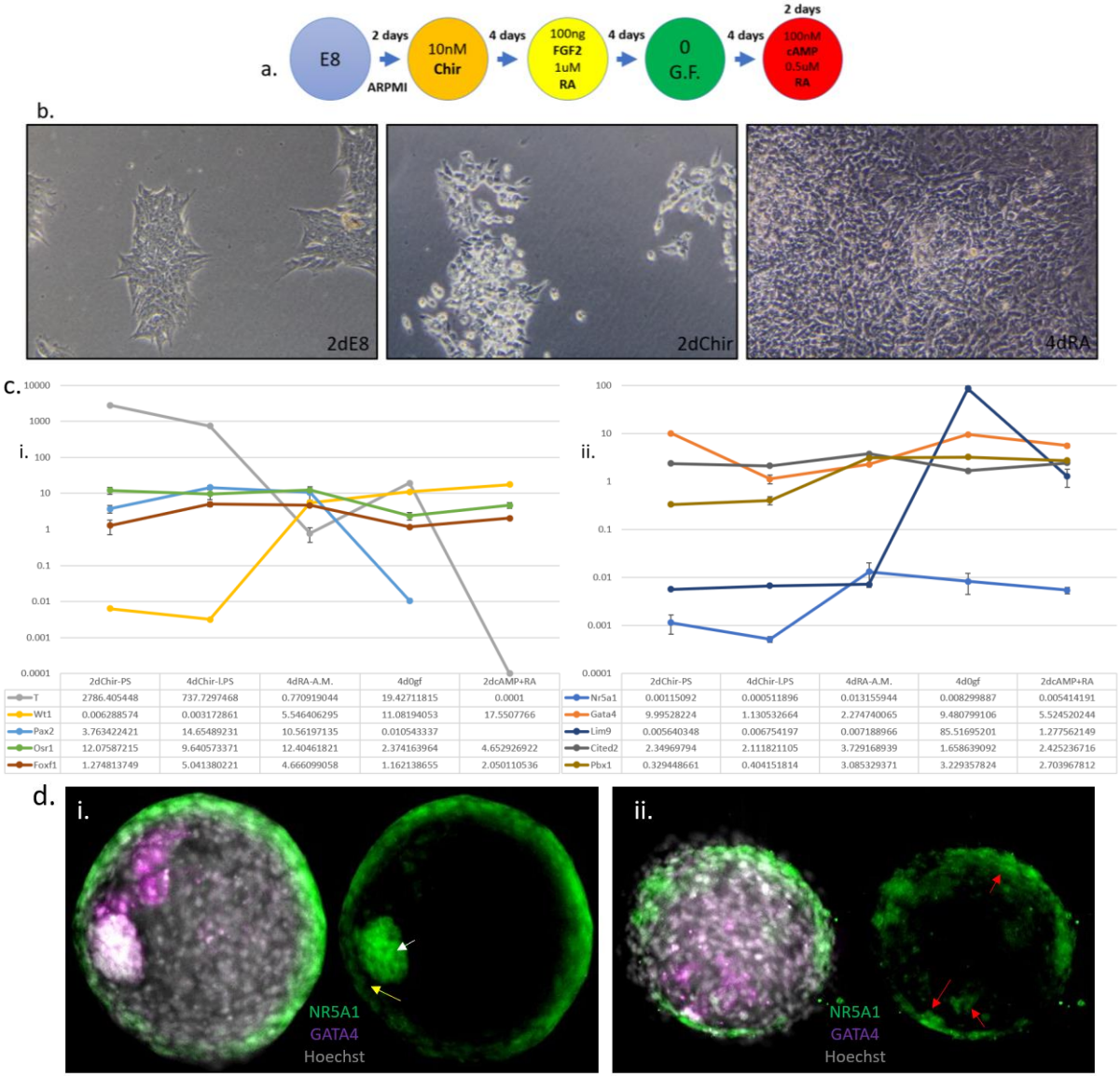


Figure.63. Adaptation of the protocol from (Knarston et al., 2020). **a**. hIPSCs were plated on VTN at $2*10^4$ cells/well of 24-well plate (~10^4 cells/cm^2) on E8 media. After two days the media was switched to E6 media with 3uM Chir supplement to reach the primitive streak (PS.). Subsequently, cells were treated with BMP4 (10ng/ml), FGF9 (200ng/ml) for mesodermal induction, and with either Heparin (1ug/ml) or RA (1uM) or both for three days; from then on the cells were cultured for three days in E6 media to achieve a bipotential gonad stage and subsequently treated for two days with 8-Br-cAMP (100nM) for steroidogenic lineage induction. **b**. Brightfield images of key points during differentiation. **c**. RT-qPCR analysis for key mesodermal genes (*Gata4*, *Wt1* and *Osr1*) and AGP related genes (*Pbx1*, *Lim9* and *Nr5a1*) compared in the three different variants of the protocol. All the results were compared with HEK293 cells, beside Nr5a1 which was compared with patient's adrenal, and normalised with Sdha expression.

In a first attempt of translating our results to hIPSCs, we used an already published protocol focusing on bipotential gonad as a basis (Knarston et al., 2020). The protocol was slgihtly modified with different combinations of RA at the mesodermal step of differentiation (**Figure.63a**). In their protocol they present a careful gradient and time analysis of the factors affecting differentiation, which we took advantage off to tailor conditions favouring high levels of *NR5A1* expression. We also added an extra step of 8-Br-cAMP, to see if we can further boost *NR5A1* expression and AP lineage specification. Subsequently, we analysed and compared the outcome of three variants of the protocol by RT-qPCR. We also took in consideration the step after Chir treatment (PS), and during the analysis we compared the genes expression of our samples with samples which we knew that they had significant expression of the genes in qPCR analysis (**Figure.63c**). The mesodermal lineage marker *OSR1* peaked at an earlier stage in our hands than in their protocol, which was followed by relatively similar *GATA4* timelines in the protocols without RA. On the other hand, conditions with RA caused a sharp increase of *WT1* expression after mesodermal induction and kept increasing. Regarding AGP related markers, we observed a steady upregulation of *NR5A1* in RA conditions, and the beginning of *LIM9* expression from the exit of termed bipotential gonad stage in RA conditions, which was further increased with the addition of 8-Br-cAMP. *PBX1*, a gene associated with the initiation of *NR5A1* expression, was used also as an AGP fating marker. All three variants significantly expressed *PBX1* after their exit from the mesodermal stage and maintained strong expression at the bipotential gonad stage, and at the step of 8-Br-cAMP induction RA treated cells kept the same expression level, while Heparin treatment reduced it by half. Taken together, we were unable to precisely replicate the published results with our hIPSC cell line, but managed to have the expression of key genes upon modifying their conditions.

In parallel, since we had promising results with the mESCs differentiation protocol, we tried to adapt the protocol to the hIPSCs line. In theory, key developmental aspects should be preserved between mouse and human pluripotent cells, but modifications may be needed to adjust to the human development profile and timing. We kept the steps till the mesodermal induction similar with minor alterations, and we added a zero-growth factor step (0GF) as in the previously used protocol (**Figure.63a**). A further induction with 8-Br-cAMP and RA aiming to boost the steroidogenic lineage was performed, resembling the forced differentiation protocol which was used for **Figure.22** (**Figure.64a**). We first examined the correct fating of the cells toward the mesodermal lineage (**Figure.64c-i**). We observed a nice induction of T indicating PS formation. Surprisingly, *OSR1* was also upregulated at this early stage, but decreased after the mesodermal fate. Early expression was also observed for LPM marker *FOXF1* and the IM marker *PAX2*. All three of these genes declined after the exit from the mesodermal stage. The earlier expression of these markers may indicate a more rapid early differentiation of human cells, when compared to mice. *WT1* was upregulated at the mesodermal stage and further increased during culture. These results show that the cells acquired a mesodermal fate with markers of LPM and IM being present. While examining the early AGP related genes we can see an upregulation of *NR5A1* and *PBX1* by the end of the mesodermal stage, which was kept steady and followed up by a Gata4 upregulation and much higher *LIM9* expression. *CITED2* on the other hand did not respond (**Figure.64c-ii**).

These results are promising and give confidence that a similar methodology with the mESCs differentiation protocol can be used to achieve similar results on hIPSCs. Thus, an attempt to the ECM of the cells and follow protocol related with **Figure.25** was made. The cells were plated in both the commercial (**Figure.25a**) and on homemade ECM plates (**Figure.25b**) with the addition of the M.CM,



	2dChir-PS	4dChir-LPS	4dRA-A.M.	4d0gf	2dcAMP+RA
T	2786.405448	737.7297468	0.770919044	19.42711815	0.0001
Wt1	0.006288574	0.003172861	5.546406295	11.08194053	17.5507766
Pax2	3.763422421	14.65489231	10.56197135	0.010543337	
Osr1	12.07587215	9.640573371	12.40461821	2.374163964	4.652926922
Foxf1	1.274813749	5.041380221	4.666099058	1.162138655	2.050110536

	2dChir-PS	4dChir-LPS	4dRA-A.M.	4d0gf	2dcAMP+RA
Nr5a1	0.00115092	0.000511896	0.013155944	0.008299887	0.005414191
Gata4	9.99528224	1.130532664	2.274740065	9.480799106	5.524520244
Lim9	0.005640348	0.006754197	0.007188966	85.51695201	1.277562149
Cited2	2.34969794	2.111821105	3.729168939	1.658639092	2.425236716
Pbx1	0.329448661	0.404151814	3.085329371	3.229357824	2.703967812

Figure.64. Adaptation of protocol of the mESCs differentiation protocol. **a**. hIPSCs were plated on Geltrex at $3*10^4$ cells/well of 24-well plate (~$1.5*10^4$ cells/cm^2) on E8 media for two days, where after the media switched to ARPMI media with 10uM Chir treatment followed up, then the cells were treated with RA(1uM) and FGF2 (100ng/ml) for four days; from then on the cells were let for four days on plain ARPMI media and after treated for two days with 8-Br-cAMP (100nM) and RA (0.5uM). **b**. Brightfield images of key points during differentiation. **c**. RT-qPCR analysis for i) key genes leading to mesodermal fate (T, *PAX2*, *FOXF1*, *WT1* and *OSR1*) and to a more AGP-CE one (*GATA4*, *CITED2*, *PBX1*, *LIM9* and *NR5A1*); the results are from two experiments. All the results were compared with HEK293 cells, beside Nr5a1 which was compared with patient's adrenal, and normalised with *Sdha* expression. **d**. IF staining for NR5A1 (green), GATA4 (magenta) and Hoechst (grey) from cells plated in **figure 25-a**. These cells have been growing for 7 days in cE6 media with 2 days of 25% M.CM addition, on plating, in ARPMI; and treated for 2 days after the plating day. i) 100nM 8-Br-cAMP + 100ng/ml FGF9 on Tenascin peptide-2 + Chondroitin, ii) 100ng/ml FGF9 on Osteopontin peptide + Heparin, IF staining for NR5A1 as single channel is presented also. The white arrow indicates specific staining for NR5A1, while red arrows indicate unspecific staining and yellow one is not clear. Both pictures have the same scale (10x magnification).

after 2 days of 0GF this time (**Figure.64a**). Furthermore, same treatments and media were used as in **Figure.54-56**. In both cases they did not remain in 2D, but instead the cells started to incorporate the ECM and folding together with, which resulted in the creation of either big 3D structures or smaller isolated ones, depending on the ECM. The analysis of this data was difficult and unsuccessful in general, but we were able to analyse samples from the commercial ECM plate one (**Figure.25a**). The

cells were stained once again for NR5A1 and GATA4, and while there were GATA4 cells positive cells, NR5A1 expression was not found. One exception was a colony on Tenascin peptide-2 + Chondroitin ECM in the presence of 8-Br-cAMP + FGF9 that was positive for both markers (**Figure.64d-i** compare with **Figure.64d-ii**).

3. Discussion and Conclusions

In this study four different mouse ES and one human IPS cell lines were used with the aim of differentiating them towards an early steroidogenic adrenocortical fate. The approach aimed to replicate the *in vivo* environment as faithfully as possible according to the available knowledge. I managed to differentiate the PSCs to a mixed lateral plate and intermediate mesoderm population with all the cell lines I worked with during this study. Even though the core protocol for differentiation remained the same, alterations had to be made to achieve this fate of the cells on each PSC line. I observed that the initial seeding concentration of cells and the treatment with Chir are the crucial steps as they affect survival of cells exiting PS and thus properly entering the mesodermal fate. During the optimization of the protocol, I observed an unusual RNA expression of the ACTH receptor (*Mc2r*) in the mouse ESC lines, which was later validated by single cell RNA sequencing performed by our collaborators on E9 and E10.5 mice. It seems that *Mc2r* has a diffuse expression at the caudal region of the embryo where the AGP is formed, which later becomes specified to develop into AP and fetal adrenal. The expression of *Mc2r* was not functional according to my data and was restricted to RNA level at this stage. However, given the specific expression, we speculate that *Mc2r* can serve as an early reporter for the switch of mesodermal cells towards the AGP fate. I therefore created an ESC line carrying a heterozygote *Gfp* knock-in to the promoter region of *Mc2r*, with the purpose of tracking and isolating these cells. Although these cells were reaching the appropriate mesodermal fate, they exhibited high cell death, which likely also affected their proper differentiation. However, transient transfection experiments with an *Nr5a1* expression plasmid demonstrated that GFP can be activated in these *Mc2r-Gfp* cells. Thus, further optimisation should be performed to allow using this tool for screening purposes but is needed on their cultured conditions.

Meanwhile, starting from the A.M. stage of the mouse cells, I screened for the effect of the most prominent signalling pathways in the embryo based on their spatiotemporal effect on the forming AGP. In the beginning I followed the approach most frequently used in published works, where the cultured cells are treated with a combination of different factors. I have tested different combinations of RA, WNT, BMP, FGF and HH signalling pathway inductors and repressors, with the purpose of inducing the *Nr5a1* expression on the mRNA and protein level. Introduction of SHH at low concentrations to the culture medium was the only promising of these treatments since it seems to maintain the low-basic expression of *Nr5a1*. Nevertheless, IF and RT-qPCR tests showed that this approach was not giving the desired high levels of NR5A1 expression. I therefore decided to grow cells on specific ECM. Geltrex is a mix of ECM proteins that is good for the general culture of cells due to its rich environment but is probably not the best suited for AGP differentiation. Indeed, the cells started to express NR5A1 after I passaged them to new ECMs, this effect further increased when the passage was done with the addition of M.CM. Probably this way of the soft 'weaning' (gradual change of culture medium) of the cells is crucial when they are plated onto the new ECM. Indeed, this gradual change is likely to better resembles more the *in vivo* situation, where everything changes are not sudden and also depend on gradient s during the development and not sudden. After testing several ECMs, I concluded that FN1 is the most suited for this differentiation based on the frequency of NR5A1 positive colonies, and reproducibility of experiments. This observation agrees with studies that show the presence of FN1 in fetal (Chamoux et al., 2001, 2002) and adult (Otis et al., 2007) adrenal cortex, but also its potential implication in the physiological development of the tissue (el Zein et al., 2019).

Based on these studies, the presence of FN1 can be either promoting the fetal adrenal niche or supporting the endothelial cells, which in turn interact with the fated AGP cells, or both. Indeed, when in FN1 culture, there is a layer (carpet) of cells bellow the forming colonies, who have the morphology of endothelial cells and appear to be positive for CD31 via IF (data not shown).

After careful screening on the new ECM for better conditions leading to steroidogenic progenitor cells, two media stood out, ARPMI and a homemade E6 medium. In general, these two media are quite different: ARPMI is a medium that reinforces the differentiation of cells towards specific fates, while E6 is a more basic and flexible medium. The most notable difference between the two is the addition of a lipid rich BSA component (AlbuMAX® II) in ARPMI. On the other hand, in the cE6 medium there is the addition of linoleic and lipoic acid, two fatty acids which are involved in metabolic pathways (Cao et al., 2018; Sprecher, 2000). Interestingly, these two media showed opposite results when cells were treated for the TGFβ signalling pathway. Under the same conditions, cells in ARPMI were able to express at higher frequency NR5A1 when the TGFβ signalling pathway was inhibited. No significant change was observed when BMP4 was added. The opposite effect was visible in cE6 medium, with BMP4 having a beneficial effect on NR5A1-positive colonies, whereas inhibition of TGFβ signalling dramatically reduced the positive colonies in culture. Thus, this indicates that ARPMI induces the TGFβ signalling pathway in this culture protocol, and certain levels of TGFβ signalling are paramount for the differentiation of the cells to NR5A1-positive ones.

During all the screenings on ECM plates I observed that the induction of PKA pathways through the treatment with 8-Br-cAMP greatly increased the number of colonies which expressed NR5A1. The cAMP/PKA pathway is directly involved in the steroidogenesis, as it is the intracellular signal transduction pathway upon ACTH binding to MC2R (Aumo et al., 2010; de Joussineau et al., 2012; Drelon et al., 2016; Mathieu et al., 2018b). The PKA pathway has been also shown to be important for the development of the fetal gland (de Joussineau et al., 2012; Dumontet et al., 2018), although we do not know how early is is active. The results presented in this study show imply a potentially very earlyier effect of the cAMP/PKA pathway on steroidogenic cell differentiation, even before ACTH is present in the embryo or its receptor is active. Although we cannot exclude this to be only an *in vitro* observation, it is interesting to note that alternative ways exist to activate this pathway through other G-protein-coupled receptors (Moody et al., 2011). Indeed, it has been shown that the cAMP/PKA pathway can be activated in human fetal adrenal cells through other G-protein-coupled receptors with neuropeptides like PACAP and VIP (Chamoux et al., 1998; Payet et al., 2003). Interestingly, unpublished single cell sequencing data (courtesy Prof. S. Nef) suggest that the PACAP specific receptor is expressed in a portion of AGP fated cells and remains to be expressed at later stages. Additionally, PACAP is expressed in the embryo during that developmental period (Sheward et al., 1998) and it has been shown to affect the development of different tissues (Z. Xu et al., 2016). It will be interesting to investigate whether this or a similar pathway may induce the initial population of the steroidogenic progenitor cells, thus kickstarting the expression of NR5A1 and the steroidogenic profile of specific cells.

Meanwhile the correlation of SOX2/GATA4 expressing colonies located in the proximity of bigger NR5A1-positive cell clusters remains a mystery. A potential explanation would be that these are epithelial cells, but our data do not presently support this hypothesis. In the same series of experiments and under similar conditions, treatment with the HH pathway inductor resulted in a similar phenotype of NR5A1 colonies as when they were close to the SOX2 ones. At the moment we

can only speculate whether SOX2 positive cells produce HH pathway activating molecules (such as SHH) and thus boosting the fating to NR5A1 positive cells. *In vivo* gut epithelial cells produce *Shh* at around E9.5 as demonstrated by whole mount RNAscope analysis (**Figure.s6**). It is also known that SHH is important for both, the development of the fetal adrenal gland and the regulation of a progenitor cell population in the adult adrenal gland that give rise to steroidogenic cells of the cortex (Finco et al., 2018; King et al., 2009; Lerario et al., 2017; M. A. Wood et al., 2013a). Thus, the idea of HH signalling affecting the fate of steroidogenic progenitor cells seems convincing. The connection with SOX2 population must of course be further tested. At this point it is important to mention that expression of NR5A1, as evidenced by IF analysis, has so far been mainly observed with R1 cells. Although I had rare IF staining of NR5A1 with Bl6 and *Nr5a1-Gfp* cells in some of the tested conditions, and potentially in the hIPSCs, it was not a recurrent event. This may be because I have spent most of the time optimising the differentiation protocol with R1 cells, and thus further optimization for other cell lines may be required.

When I firstly tried to make aggregates from my cells at the A.M stage, I was unsuccessful in promoting any steroidogenic profile in these samples. Although I was able to collect the information that RA promotes *Mc2r* expression while Chir inhibits it, which is an information that is useful when looking to make functional ACTH-responsive cells. This drastically changed when I formed aggregates from R1 and Bl6 cells under the refined conditions that I used for replating the A.M. cells on new ECM. This can be also linked with the importance of the ECM at this stage, since it has been shown that FN is paramount in cell clustering and adhesion (Beyeler et al., 2019; Cseh et al., 2010; Radwanska et al., 2017). Furthermore, the reduction of the number of seeded cells seemed to benefit the differentiation towards steroidogenic lineage. In the bigger picture, in the first set of my experiments I was able to create aggregates which express *Nr5a1* in significant amounts when compared to an E12.5 adrenal gland. This was coupled with the upregulation of early steroidogenic genes like *Star* and *Cyp11a1* for R1 and Bl6 cells, while in some experiments with R1 cells I had also significant expression of the late steroidogenic genes *Cyp21a1* and *Cyp11b1*. Additionally, I observed a pattern where a certain level of *Mc2r* expression it was coupled with the expression of steroidogenic genes. In some samples I also observed a significant increase on the expression of *Mgarp*. This expression is interesting, since the preliminary single cell RNA sequencing data suggest that the AP cells express high amounts of *Mgarp* this gene and in more frequency, when compared to the rest of sampled cells. On the other hand, I observed that cells in ARPMI medium favoured fating towards the GP when compared to cE6, and that Bl6 cells were expressing more *Foxl2* and *Fst* when compared with R1 cells under similar conditions.

By focusing on R1 cells, I was able to confirm the induction of steroidogenic genes (*Nr5a1, Star, Cyp11a1, Cyp21a1*) in the sphericalplates and in ULA plates, under 8-Br-cAMP treatment (cAMP/PKA pathway induction) in the commercially available E6 medium. Thus, I was able to create aggregates with an active adrenocortical steroidogenic profile, following this differentiation protocol and not relying in forced *Nr5a1* induction. The induction of the steroidogenic profile was once again greater in sphericalplates than ULA plates, and as I indicated, the results can be improved even more with optimization of the protocol. Nevertheless, the repetition of the results with commercial and standardized materials opens the path for broader use of the protocol. This is complemented with the steroidomics results, which even though they come from an individual experiment, they ensure the functionality of these cells. It is important to have in mind that we do not know the percentage of the cells which have this active steroidogenic profile. Furthermore, it is yet inconclusive the steroidogenic

state of the aggregates at the endpoint of the experiment. Since the steroidogenic identity of these cells has been confirmed, their detailed functionality should be the next step.

The activation of cAMP/PKA pathway was beneficial in both, 2D and the 3D experiments. This is clear based on the IF results from 2D and the higher expression of steroidogenic genes in aggregate experiments. More unclear is which other factors would benefit the steroidogenic fate and push further for functional adrenocortical steroidogenic cells, which in turn could be transplanted or used for functional analysis. The cE6 medium had delivered the best results (Kug1) and it seems to favour more the AP fate, but the results were not constant. This seems to be equilibrated with the use of the commercially available E6, in which the cells differentiated successfully in 2/2 sphericalplate and 4/4 ULA plates experiments. The E6 medium is less restrictive and allows more flexibility on guiding the cells through signalling cues. Thus, strict monitoring of correct fating of cells at each stage is required to ensure proper differentiation towards the steroidogenic identity. This is something that can be used in our advantage though, since we have seen the plasticity of these cells, and their responsiveness to signal ques in this medium.

4. Future perspectives

In this study I hypothesized, based on my *in vitro* results, possible implications of signalling pathways in the fating and differentiation of cells towards steroidogenic lineage. In future experiments it will be important to test these hypotheses. Normally NR5A1 promotes the expression of MC2R, which then will transmit the ACTH signal, thus activating the cAMP/PKA pathway and keeping up the expression of NR5A1 (Angelousi et al., 2000; Frigeri et al., 2000; Hammer et al., 1999; Kulcenty et al., 2015; C. M. Wang et al., 2017; W. H. Yang et al., 2010). Although the initial activation of *Nr5a1* at the early adrenocortical progenitor cell population has been attributed to its enhancer element (Zubair et al., 2006a, 2008a), the signalling cues and pathways involved in this initial trigger have not yet been discovered. For me the most intriguing aspect is a potential contribution of PACAP to this initial activation of the steroidogenic profile of the cells. The fastest way to test this hypothesis would be to stain mouse embryos at the age of E9.0 until E10.5 for the receptor of PACAP (ADCYAP1R1) coupled with GATA4 and NR5A1. Alternatively, knock-out mice that have been created previously should be analysed for the effect of PACAP deletion on adrenal gland development. This hypothesis could also explain the early expression of *Mc2r*, both *in vivo* and *in vitro*, despite the absence of its co-receptor *Mrap* (Novoselova et al., 2018). Both PACAP and ACTH through their receptors can potentially activate the cAMP/PKA pathway, thus triggering the transcriptional activation of the steroidogenic profile on the properly fated cells. It will be interesting to stimulate cells of A.M. stage with PACAP or use it instead of 8-Br-cAMP at these early steps of the protocol and observe if they have similar effects on the steroidogenic profile of the cells.

Another interesting pathway for further examination is HH signalling. As mentioned before, combining the *in vitro* preliminary data and the *in vivo*, we can speculate that a similar recruiting effect like in adrenal capsule (Dörner et al., 2017) may be happening in the CE for the AGP cells. In principle this can be tested using lineage tracing analysis using a tamoxifen inducible Gli*1-CreERT2* line in combination with a reporter strain. Tamoxifen induction should be targeted to the developmental window of E8.5-10.5, in order to capture the early AGP-AP fating. Furthermore, since the late *in vitro* differentiation stage (especially in 3D) shows a steroidogenic transcription profile, a proper testing with the inclusion of SHH shall be done which will imitate the *in vivo* development of the fetal cortex at around E12 (King et al., 2009; Vidal et al., 2016). Maybe the inclusion of SHH in the pool of factors used in the final stages of differentiation protocols (so far) will promote more cells towards the steroidogenic profile or/and lead to a further maturation. In parallel, deciphering the role of the TGFβ pathway on the fating of the steroidogenic progenitors should be quite revealing. In my data, the outcome of the treatments for TGFβ signalling strongly depended on the medium used for cultures (cE6 or ARPMI). This may be an indication for the influence of TGFβ signalling on the proper modulation of this differentiation protocol *in vitro*, but potentially also *in vivo*. Indeed, it has been already shown that TGFβ can interfere with steroidogenesis (Brand et al., 1998; Mattar et al., 2020; Park et al., 2014; Stelcer et al., 2020b; X. Y. Zhang et al., 2019); it can also be affected by the ECM (Hinz, 2015). A future approach would be to do a systematic screen for activation and inhibition of TGFβ signalling in both the 2D and 3D system, and properly characterise the effects of the treatments on the fate of the cells. This may unveil a signalling network that supports the desired differentiation to AP cells and the support of their steroidogenic profile. Testing this theory *in vivo* will be more challenging as TGFβ signalling is highly complex with a multitude of signalling molecules and receptors.

A tissue specific knockout for all ALK receptors within the CE or early AGP will therefore be difficult to achieve. Alternatively, the negative effect of the pathway could be explored by tissue-specific mutagenesis of the receptors, with the purpose of overactivating them and observe if this constant activation of the pathway inhibits the formation of steroidogenic cells.

Furthermore, I firmly believe that tailoring the ECM more like the fetal adrenal can improve the outcome of the differentiation. There can be the argument that if the differentiation goes as planned the cells should create their own ECM, but in cases the ECM can be provided by cells of different origin of the desired population. For example, in the human fetal adrenal gland, the FN1 and laminin seem to come from blood vessels and from small arteries respectively (Chamoux et al., 2001). Thus, the creation of the optimal niche for the differentiation of the cells depends on multiple cell types. Providing the ECM artificially to recreate this environment *in vitro* will therefore be beneficial for a more controlled and successful differentiation. As I mentioned, laminin, and Collagen-4 are good candidates as they are both detected in fetal adrenals. It will be interesting to know in more details what is the proper ECM for each stage of the adrenal cortex so that we can properly modulate the concentrations of the ECM compounds. This should also prove beneficial for primary cultures, as the ECM can provide an environment closer to the *in vivo* context and the cells will be less 'shocked' when moved into culture, thus increasing the viability of such cultures for future experiments.

Lastly, the 3D assembly (aggregates) part of this study has shown the highest potential in generating cells with steroidogenic profile, based on the RT-qPCR and the steroidomics analysis. As I mentioned multiple times, refinement and further establishment of the protocol is necessary, but this should not stop its evolution. It is equally important to take this working protocol a step further by addressing the cells functionality *in vivo* by transplanting these aggregates in mice adrenals. In this occasion, a general labelling marker for the cells will be sufficient to see their integration to the tissue, and then later their sorting from the rest of the cells, where we could be able to see their commitment to the steroidogenesis. Furthermore, a reliable marker of steroidogenesis will be needed for future polishing of the protocol, since as we mentioned, we do not know the percentage of the converting cells and we do not have a practical way to isolate these steroidogenic cells. Also, a more careful analysis in what makes the sphericalplates more successful, in terms of steroidogenic profile induction in this protocol, has to be analysed in further detail. This could give insights not only on how to improve this *in vitro* protocol, but also potentially on the development of the adrenocortical tissue. It will be of great interest to study this protocol with hIPSCs and if it is translatable to these cells.

A final remark regarding in *vitro* differentiation: from my experience the outcome of those experiments is sometimes unpredictable (failing) even though the protocol is followed religiously every time. This indicates that minor changes happen in the culture that are presently hard to control and difficult to detect at early stages of the experiment. Sadly, this costs time and resources, and it will be useful if we could easily appraise the progress of the differentiation protocol and the differentiation into steroidogenic progenitor cells. Presently, we are using either IF staining or RT-qPCR to detect differentiation, and we need to narrow down additional markers that are implicated in the fating and not only the identity of the cells. Visual screening methods such as the *Mc2r*-GFP line should improve the speed with which different conditions can be optimized. Finally, machine learning (ML) and deep artificial networks (DL) may be applied to our *in vitro* differentiation systems and may provide additional information to improve protocols, as they have already started to be applied in biological research (Gómez-de-Mariscal et al., 2021).

In summary, in this study I present an *in vitro* system which has the potential to differentiate into steroidogenic cells following a step-by-step approach, responding to physiological signalling cues. Importantly, I have shown that this protocol can produce NR5A1 positive colonies under specific conditions with R1 mESCs, something which has not been shown in the literature without exogenous source of NR5A1 as a trigger. Furthermore, I showcased the importance of the proper ECM and niche for successful targeted differentiation, and the potential of applying this protocol in other mESC lines and hIPSCs. cAMP/PKA activation was identified as a pathway that clearly promotes steroidogenic differentiation at this early stage, *in vitro*. Based on the 3D assembly results, we have steroidogenesis and the next step to validate this protocol will be a functional analysis of the cells/aggregates; quantification of these cells is also paramount. Due to the potential of this protocol, translation and optimization of the protocol in hIPSCs should be done. Additional studies should be performed to further characterise the HH and TGFβ pathways, as my preliminary data suggest them to impact the formation of steroidogenic progenitors at least *in vitro*. The *Mc2r-Gfp* line created in the course of this theses may help to perform these studies and further optimize conditions to develop a highly reliable and efficient *in vitro* differentiation protocol.

5. Materials and Methods

5.1. Methods and protocols

mESCs maintenance

For the *in vitro* experiments R1, C57/BL6 and mixed background ESCs were used. All mouse cell lines (unless it is said otherwise) were maintained in the presence of inactivated feeder cells, isolated from mouse embryos at E12.5-13 as it has been described by established protocol (Bryja et al., 2006). The mESCs where then cultured in K.O. DMEM medium containing 15% K.O. serum, supplemented with Glutamax, Non-Essential amino acids, 2-Mercaptethanol and Lif. The pluripotency was maintained with the 2i systems of PD0325901 and CHIR9902. For passaging, the medium was washed with DPBS and Trypsin was added to the cells. After disassociation, trypsin was deactivated with 1:1 NBCS and the cells were centrifuged in 1200 rpm of 4 min and then replated on ES media.

Differentiation of mESCs to mesodermal cells

Plates were precoated with 1% Geltrex in DMEM-F12 at 37^{o}C. ESCs were seeded at a ~3*10^{4} cells/cm^{2} (24 well plates were used predominately), in 1:1 ReproFF2 and MEF CM, with 10ng/ml Activin A and 10ng/ml FGF2. The MEF CM is the mESCs maintenance medium gathered from MEF culture. This can be replaced with mESC medium lacking the 2i and Lif, depending on the fragility of the cell line. After two days in the media, the cells reach EpiSC state. Thereafter, the EpiSC media is washed away with DPBS once and replaced with ARPMI containing 7-8uM CHIR99021, for 2 to 3 days. By the end of the treatment the cells will be at PS stage. At this point a wash with ARPMI is done to remove the dead cells and they will be further treated with 1uM Retinoic Acid and 100ng/ml FGF2 in ARPMI for 4 days; thus reaching the anterior mesoderm stage. At this stage the cells were either further treated with other factors or disassociated with Trypsin and used in other protocols. Media were refreshed every day.

Plating and differentiation on ECM plates

Wells were coated with FN1, VTN and Col1 at 1ug/ml, 200ng/ml, 4ug/ml respectively, in DPBS O/N at 4^{o}C and then brought at 37^{o}C for an hour before the plating of the cells. The medium of cells at A.M. stage was first gathered, filtrated, and kept as M.CM to be added later. The cells were then washed once with PBS and treated with 50% Trypsin in DPBS at 37^{o}C for 5 min to disassociated them. The reaction was stopped with 1:1 FBS addition, cells were gathered, centrifugated for 4 min in 850 rpm, and resuspended in M.CM at 10^{7}/ml concentration. Cells were plated on the new ECMs at 20-27*10^{4} cells/cm^{2} in the selected medium containing 25% M.CM. After two days and then every two days, the medium was replaced with the selected medium and with the addition of desired treatments as it is described at section **2.3**. Around 20% of initial medium was kept at the wells during the media refreshment.

Aggregate formation and differentiation

Aggregates were formed in either ULA plates or SphericalPlate 5D. The medium of cells at A.M. stage was first gathered, filtrated, and kept as M.CM to be added later. The cells were then washed once

with PBS and treated with 50% Trypsin in DPBS at 37°C for 5 min to disassociated them. The reaction was stopped with 1:1 FBS addition, cells were gathered, centrifugated for 4 min in 850 rpm, and resuspended in M.CM at 10^7/ml concentration. Cells were places in wells at a concentration as described at section **2.4**, according to their plate also, to form the spheroids. For the SpericalPlate we made sure that there no bubbles in the well beforehand, by centrifugating the plate with half medium per well for 1min at 2500 rpm. In SphericalPlates the aggregates were formed normally without centrifugation, due to the architecture and technology of the plates. In the ULA plates, the cells were centrifugated for 2 min at 900 rpm to help them form aggregates. The aggregates were initially seeded in the selected medium (ARPMI or cE6) containing 25% M.CM, which was toped up with only the selected medium after 2 days. For further refreshment of the medium, half of the media was removed from the wells and replaced with equal volume. During the refreshment of the media treatments took place.

hIPSCs differentiation

Plates were precoated with 1% Geltrex in DMEM-F12 at 37°C. ESCs were seeded at a ~$1.5*10^4$ cells/cm^2 (24 well plates were used predominately), in E8 medium for 2 days. After, the medium was replaced with ARPMI medium containing 7-10uM CHIR99021, for 3-4 days. By the end of the treatment the cells will be at PS stage. The treatment of the cells changed to 1uM Retinoic Acid and 100ng/ml FGF2 in ARPMI for 4 days. Up until here the medium was changed every day. Following the cells were let for 2-4 days with just ARPMI medium, and the medium was refreshed every two days from now on. At this point the cells were either moved to new ECM as it have been described or treated with of 0.5uM RA and 100nM 8-Br-cAMP in ARPMI.

mESCs transfection

All the transfections were done using the protocol of Lipofectamine™ 3000 Transfection Reagent kit (**Ref** L3000001, Invitrogen ™). For the transfection, the cells were already placed on Gelatine without feeder cells from the previous day, and their transfection efficiency was assessed by the co-transfected GFP expressing plasmid, and further selection was done with antibiotics (if it was possible).

Steroidogenic program induction by transfection

The cells were transfected with mouse NR5A1 expression plasmid in pSG5 vector (courtesy of E. Lalli, Sophia Antipolis, France) (Aigueperse et al., 2001) as it was already described. The transfected cells were then cultured in full GMEM medium without Lif and 2i, with the addition of 0.5uM RA and 100nM 8-Br-cAMP. The medium was refreshed every day, and the analysis happened after two days of the treatment.

Cells used

For hIPSCs experiment HMGU1 IPS from human foreskin fibroblasts was used, provided to us by Dr. BERTACCHI Michele of Studer lab. Bl6 and R1 ES cells were provided to us by Prof. Smith group (Wellcome-MRC Cambridge Stem Cell Institute). *Nr5a1*-GFP cells were derived from mice by Dr. JIAN MOTAMEDI in our lab. Mouse embryonic fibroblasts were prepared as it is described above and after mitomycin (**Ref** M4287-5X2MG – Sigma) they were stored at liquid nitrogen. For all the freezing of the cells a mix of 1:1 of cell medium and freezing medium (25% DMSO -75% FBS) was used.

cE6 medium

The custom E6 (Essential 6) medium was made as it follows: 500ml Ham's F-12 Nutrient Mix (**Ref** 31765035 Gibco), 270 mg Sodium Bicarbonate (**Ref** S6014, Sigma-Aldrich), 7ug Sodium Selenite (**Ref** 214485 Sigma-Aldrich), 32 mg L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (**Ref** A8960 Sigma-Aldrich), 5 mg holo-Transferrin human (**Ref** T0665 Sigma-Aldrich) and 10mg Insulin solution human (**Ref** I9278 Sigma-Aldrich). All the components were mixed in the bottle of the medium and filtrated afterwards.

RNA isolation and RT-qPCR

For RNA isolation from 2D, the media were removed and washed two times with DBPS, then RLT buffer was placed directly on the cells and the whole extract was gathered by pipetting. For 3D structures, the aggregates were gathered in tubes and lightly centrifugated at 500 rpm for 5 min, media was removed and replaced with RLT buffer. The RNA isolation was followed according to the protocol of RNeasy Micro Kit from Qiagen, and RNeasy Micro Kit (**Ref** Cat. No. / ID: 74004) or RNeasy Mini Kit (**Ref** Cat. No. / ID: 74106) according to the number of the cells and the expected amount of RNA. For reverse transcription 150-400 ng of total RNA was used per sample up to 10ul in RNase free H_2O, and the procedure was done as follows. 1ul of 150ng/ul random primers (**Ref** 48190011 - Invitrogen ™) and of 10nM dNPT mix (**Ref** 18427013- Invitrogen ™) were added and heated up at 65^{o}C for 5 min, and then placed on ice to cool down. A mix of 4ul of 5X single-stranded buffer (**Ref** 18080093 - Invitrogen ™), 2ul of 0.1M DTT (**Ref** 18080093 - Invitrogen ™) and 1ul (40 units) RNaseOUT (**Ref** 10777019 - Invitrogen ™) was added to the samples, briefly mixed, spined down and incubated for 10 min at 37 ^{o}C. At the end of this short incubation 1ul (200 units) of Reverse Transcriptase (M-MLV RT) (**Ref** 18080093 - Invitrogen ™) was added, the samples were gently mixed and spined down. Subsequently, the samples were incubated at 37 ^{o}C for 50 min to let the enzymatic reaction take place and then the enzyme was disactivated by 15 min incubation at 70 ^{o}C.

For the qPCR, the 20ul of samples were diluted 1/5 at 100ul, after cooling down them down and spined down. From these samples, 1.5ul was added to a mix of 5 ul of SYBR Green I Master mix (**Ref** 04707516001 – Roche), 0.06 ul of each primer (10uM) and 3.38ul of H_2O (**Ref** 04707516001 – Roche), in a 96-well compatible plate (**Ref** 4Ti-0952 – Dutcher). LightCycler® 480 System (Roche) was used for the reading of the plates. A 40 cycles program was used with 60^{o}C annealing temperature.

Immunofluorescence for cells

Medium was removed from the cells, and they were fixed with 4% PFA (diluted in PBS) for 10-15 minutes. PFA was replaced with PBS and the samples were let at cold room O/N. Thereafter PBS was removed and 30 minutes permeabilization with 0.5% Triton (in PBS) was done in RT. The samples were washed two times for 15 minutes each with PBS and continued with incubated them with 4% BSA + 3% NDS + 0.05% Triton PBS for 1 hour in room temperature, to block the unspecific bindings of the antibodies. Following up, the cells were incubated with the corresponding primary antibody diluted accordingly 4% BSA + 3% NDS + 0.05% Triton in PBS, overnight at 4^{o}C. The next day, the coverslips were washed two times with PBS in order to remove any excess antibody, for 15 minutes each time, and incubated with Hoechst and the corresponding secondary antibody diluted 1/500 in PBS for 1 hour at RT. From this stage on, the coverslips were protected from light to prevent any loss of fluorescence. When the incubation was done, the coverslips were washed two times with PBS for 15 minutes each. Lastly, the PBS was replaced with 50% glycerol in PBS, for imaging and for maintenance in 4^{o}C.

IF on tissue slices

Embryos were dissected and placed in PBS, before fixating them for O/N at 4°C in 4% PFA (**ref**)/PBS freshly prepared. The PFA was washed two times with PBS for 15 min at RT. Thereafter, gradual dehydration of the embryo was performed through 20%, 4O%, 60%, 80% Ethanol/PBS (30min), and the samples were then stored in 70% Ethanol at 4°C. Samples were then prepared for paraffin embedding in dehydration station (LEICA TP1020) and embedded the day after at the embedding station (LEICA EG1150). The samples were cut in 5 um sections shortly before the experiment and they were dried O/N at 37°. The samples were then rehydrated in Xylene for 2x 5 min and with successive incubations 100,90,70 and 40% of ethanol solutions for 3 min each and washed with distilled H_2O. The slides were then placed in whistling pressure cooker (already whistling) containing 1.9L Milli-Q H_2O and 100ml 20x Sodium citrate for 2min, and then it was cooled down at 4°C. The rehydration was also done using PT Link (Agilent) in either pH6 or 9 depending on the antibody, followed up by one wash in PBS for 15 min; in these results pH6 was used. The samples were blocked for unspecific antibody binding with 10% NDS, 3% BSA, 0.1% Tween20 (**Ref** P1379 Sigma-Aldrich) in PBS, in RT for 1h. Blocking buffer was replaced with a 3% NDS, 3% BSA, 0.1% Tween20 PBS buffer, containing the selected primary antibodies at proper dilution, and incubated O/N at 4°C. The next day, the primary antibodies were washed 3 times with PBS for 10 min each and incubated with corresponding secondary antibodies in antibody buffer containing Hoechst 1/500 for 1h in RT (from here on they were protected from light). Finally, the secondary antibodies were washed again 3 times with PBS for 10 min each and the samples were mounted with 50% glycerol in PBS, for imaging and for maintenance in 4°C.

RNAscope for tissue slices

Embryos were fixed in 4% PFA O/N at RT and embedded in paraffin as described and they were cut in 5 um sections shortly before the experiment. The sections were dried O/N at 37° and heated for 1h at 60° the next day. The paraffine was then removed with 2 incubations at Xylene for 5 min, followed up by 2 baths in 100% Ethanol for 3 min to dehydrate the tissue. H_20_2 was performed in RT for 10 min and then washed away with distilled H_2O, antigen retrieval can be done at this point, but must be washed with 100% Ethanol after. From here on the normal protocol of RNAscope with its buffer has been used (**Ref** 322350-ACD).

Whole mount RNAscope for E9.5 mouse

Embryos were dissected and placed in PBS, before fixating them for 1h at RT (according to the thickness of the embryo) or O/N at 4°C in 4% PFA/PBS freshly prepared. The PFA was washed two times with PBS for 30 min on ice (or in 4°C). Thereafter, gradual dehydration of the embryo (on ice or in 4°C) was performed through 25%, 5O%, 75%, 90% Methanol/PBS (15-30min), and 2 final washes with 100% methanol for 10 min each. Before hybridization, the embryos were bleached with 6% H_2O_2 in Methanol for 15-60 min (depending the thickness) on ice or in 4°C, this step can be skipped. Rehydration of embryos (on ice or in 4°C) through 75%, 5O%, 25% methanol/PBS, 15-30 min each with 2x PBT (PBS buffer + 0.01% Tween-20) at the end. From now on, buffers of the RNAscope kit are used (**Ref** 322350-ACD). Digestion of the embryo with 2 drops of Pretreat 3 in RT for 3-5 min was followed with wash of the tissue 3 × 5 min in PBT at RT on rocker to stop the digestion. At this point we can start to pre-warm probes to 40°C (10min) and then cool down to RT (~ 10min) to ensure there is no precipitation. The robes were let to hybridize at 40°C O/N. Post hybridization, the embryos were washed 3 times with 0.2× SSCT (saline-sodium citrate buffer + 0.01% Tween-20) 1 ml in RT for 15 min,

followed by post-fixation in 4% PFA/PBS for 10 min in RT. 3 more washes with 0.2× SSCT were followed by 2 drops of Amp1 in 40°C for 30 min to pre-amplify the hybridization. The Amp1 buffer was washed away with 3 washes with 0.2× SSCT, and Amp2 was added to the samples for 15 min in 40°C as a signal enhancement. Once again, the samples were washed with SSCT and Amp3 was introduced to the samples for 30 min in 40°C as an amplifier of hybridization and washed away again with SSCT. A consecutive treatment of Amp4 to 6 buffers was followed for 15 min each in RT and protected from light, with SSCT washes between them. To reveal the signal of the probe 1:60 ratio of Fast red-b to Fast red A was mixed well and incubated with the embryos for 10min in RT. To remove the excess colouring rinse with tap water twice for 2min (Counter stain with Hoechst: SSCT (1:10000) 4°C O/N or 1h RT). From here on, further whole mount IF staining can be performed.

H&E staining

Paraffin sections of 5uM were incubated in Xylene for 2x 5 min and rehydrated in successive ethanol solutions from 40 to 100%. After a quick wash with distilled water, slides were then incubated for 3 min in hematoxylin solution, thoroughly rinsed in water (10 min) and incubated in eosin solution for 3 min. Successive dehydration in ethanol solutions was done up to 100% and the slides were incubated once again 2 min in Xylene before mounting. The slides were let to dry O/N under the fume hood.

Mice used

If not otherwise stated, all lines were maintained and bred on mixed genetic background. Previously reported mice lines *Nr5a1-Cre* (Bingham et al., 2006) and *Bmp4*^{flx} (W. Liu et al., 2004) were put in mating, with mixed background agouti mice. For the WT studies CD-1 and NMR1 mice (pregnant and not) were used.

SphericalPlates 5D were purchased from **Kugelmeiers** Ltd

The commercial screening ECM plates were purchased **denovoMATRIX** GmbH

Proteins are written with all uppercase for both human and mouse (ex: NR5A1 protein). Genes are italicized and written in all capital letters for human (ex: *NR5A1*) and with only the first letter in uppercase and the rest in lowercase for mouse genes (ex: *Nr5a1*).

5.2. References of materials used

Table of factors used in the *in vitro* cell culture experiments:

Reagent	Supplier
CHIR99021	SML1046-Sigma
8-Bromo-cAMP, sodium salt	1140-Tocris
Activin A	GFH6-interchim
BMP6	120-06-Peprotech
CD437	72724-Stemcell Tecnologies
Collagen I, Rat	11519816-Corning™
DMH-1	4126-Tocris
Dorspmorphin	Dor-02-StemRD
Forskolin	F6886-Sigma
Human Activin A	GFH6-CellGS
Human FGF acidic (FGF-1)	100-17A-Peprotech
Human plasma FN1	10526961-Corning™
IWP-2	6866167-Peprotech
PD0325901	S1036-Selleck Chemical
Purmorphamine	72204-Stemcell Tecnologies
Recombinant Human BMP-4	120-05ET-Peprotech
Recombinant Human BMP-5	120-39-Peprotech
Recombinant Human BMP7	354-BP-010-R&D
Recombinant Human FGF-8a	100-25A-Peprotech
Recombinant Human FGF-9 Protein	273-F9-R&D
Recombinant Human FGF-basic (FGF2)	100-18B-Peprotech
Recombinant Human IGF-I	100-11-Peprotech
Recombinant Human IGF-II	100-12-Peprotech
Recombinant Human Noggin	120-10C-Peprotech
Recombinant Human R-Spondin-1	120-38-Peprotech
Recombinant Human R-Spondin-3	120-44-Peprotech
Recombinant Human sDLL-1	140-08-Peprotech
Recombinant Human sDLL-4	140-07-Peprotech
Recombinant Human Sonic Hedgehog/Shh	1845-SH-RnDsystems
Recombinant Human Sonic Hedgehog/Shh Protein, High Activit	8908-SH-RnDsystems
Recombinant Human/Murine/Rat BMP-2	120-02-Peprotech
Recombinant Murine Wnt-3a	315-20-Peprotech
Retinoic acid	R2625-Sigma
SB4	6881-Tocris
SB590885	S2220-Selleck Chemical
Synthetic Retinoid ec23	AMS.SRP002-2-AMSBIO
Vitronectin Recombinant Human Protein	A31804-Corning™
XAV939-1mg	72672-Stemcell Tecnologies
Y-27632 dihydrochloride	1254-Tocris

Table of reagents and media used in cell culture:

Reagent	Supplier
Advanced DMEM / F-12	12634010-Gibco™
Advanced RPMI 1640 (ARPMI)	12633012-Gibco™
Cell culture plates	Falcon®-Corning
DMEM KnockOut ™	10829018-Gibco™
DMEM/F-12	11320033-Gibco™
DPBS	14190250-Gibco™
Fetal Bovine Serum (FBS)	A3840402-Gibco™
Geltrex	A1413302-Gibco™
Glasgow MEM (GMEM)	11710035-Gibco™
GlutaMAX ™	35050061-Gibco™
Ham's F-12K (Kaighn's)	21127022-Gibco™
Ham's F-12K NutriMix	11765054-Gibco™
KnockOut ™ Serum Replacement	10828028-Gibco™
LIF	ESG1107-Sigma
MEM - Minimum Essential Media	41090036-Gibco™
MEM non-essential amino acid solution	11140050-Gibco™
Newborn Calf Serum (NBCS)	16010159-Gibco™
ReproFF2	RCHEMD006-REPROCELL
RPMI 1640	11875093-Gibco™
STEMdiff™ APEL™2	05275-STEMCELL™
TeSR™-E6	5946-STEMCELL™
TeSR™-E8™	5990-STEMCELL™
Trypsin-EDTA	25200056-Gibco™

Table of chemical reagents:

Target	host	reference	supplier
PAX2	rabbit	71-6000	invitrogen
Brachyury (T)	goat	AF2085	R&D
GATA-4	goat	sc1237	santacruz
GATA-4	Mouse	sc-25310	Santacruz
LIM1	rabbit	NBP2-68955	novus
NR5A1	rabbit	18658-1-AP	proteintech
Oct-3/4	Rat	MAB1759	R&D
SOX17	goat	AF1924	R&D
Sox-2	goat	sc-17320	Santacruz
WT1	goat	AF5729	R&D

Table of chemical reagents:

Reagent	Reference	Supplier
Ethanol absolute	20821.321	VWR
Hoechst 33342	14533	Sigma
Methanol	1.06009.1000	merck
PFA 32%	50980495	Fisher
Tween 20	P1379	sigma
Xylenes	534056	Sigma

Table of human qPCR primer pairs:

Gene	Sequence
Cited2 Fw	TGCCGCCCAATGTCATAGACAC
Cited2 Rev	CAGCTCCTTGATGCGGTCCAAA
Foxf1 L	CGTATCTGCACCAGAACAGC
Foxf1 R	GACAACTCCTTTCGGTCACA
Gata4 L	CTGTCATCTCACTACGGGCA
Gata4 R	GGAGACGCATAGCCTTGT
LHX1 Fw	ATGCAACCTGACCGAGAAGT
LHX1 Rev	CAGGTCGCTAGGGGAGATG
LHX9 Fw	ACCTGCTTTGCCAAGGACGGTA
LHX9 Rev	TGACCATCTCCGAGGCGGAAAT
Mc2r Fw	CCCAGAAAGTTCCTGCTTCA
Mc2r Rev	TCTTCAGGATCTTTTCTTCCTTG
Nr5a1 L	CATCATCCTCTTCAGCCTGG
Nr5a1 R	TGGCACAGGGTGTAGTCAAG
Osr1 Fw	CCTACACCTGTGACATCTGCCA
Osr1 Rev	GTGAGTGTAGCGTCTTGTGGAC
Pbx1 L	CAGATGCAGCTCAAGCAGAG
Pbx1 R	CTCTTTGGCTTCCTCACTGG
Sdha Fw	GAGATGTGGTGTCTCGGTCCAT
Sdha Rev	GCTGTCTCTGAAATGCCAGGCA
T Fw	AGGTACCCAACCCTGAGGA
T Rev	GCAGGTGAGTTGTCAGAATAGGT
WT1 L	GAATGGACAGAAGGGCAGA
WT1 R	GACACCGTGCGTGTGTATTC

Table of mouse qPCR primer pairs:

Gene	Sequence
Cited2 L	ATCGCAAAGACGGAAGGA
Cited2 R	TGCTGCTGGTGATGATGC
Cyp11a1 Fw	TCCATTACCATCAGATGCAGA
Cyp11a1 Rev	GGGGTCCACGATGTAAACTG
Cyp11b1 Fw	TGAAAGTGAGGAGAGGACTG
Cyp11b1 Rev	CGTGGAGACACAAGAGAAAG
Cyp21a1 Fw	GATTCCTCCTTTCCAGGTGC
Cyp21a1 Rev	TCAAGGACGCTCACCCT
Fgf5 Fw	GCTGTGTCTCAGGGGATTGT
Fgf5 Rev	CACTCTCGGCCTGTCTTTTC
Foxa2 Fw	TATTCCTCCACGCGATGC
Foxa2 Rev	TTGCTCACGGAAGAGTAGCC
Foxf1 Fw	CCAAAACAGTCACAACGGGC
Foxf1 Rev	TCACACACGGCTTGATGTCT
Foxl2 Fw	GTTTCTTCCACCTTCGGAAGGA
Foxl2 rev	CCTCTAGAAAATTTGGTCCCCG
Fst L	AAGCATTCTGGATCTTGCAACT
Fst R	AAAGCTGTAGTCCTGGTCTTCCT
Gata4 Fw	TTTCTGGGAAACTGGAGCTG
Gata4 Rev	CCTTGCTTTCTGCCTGCTAC
GFP Fw	AAGCTGACCCTGAAGTTCATCTGC
GFP Rev	CTTGTAGTTGCCGTCGTCCTTGAA-3',
Lhx9 Fw	TGGGAGTGGACATCGTGAATT
Lhx9 Rev	GAAAGAAGTTCGCATCCGTTTG
Lim1 Fw	CAGGAGACTGGCCTCAACAT
Lim1 Rev	GCGCTTAGCTGTTTCATCCT
Mc2r Fw	CACAAATGATTCTGCTGCTTCCA
Mc2r Rev	GCCGTTGACTTACAGAAATCACGA
Mgarp Rev	AGCAGTGTCTCCAGGCTCTGAA
Mgarp Fw	CATCAAAGCAAGTGAGACGTACAG
Mrap Fw	TTCGTGGTGCTCCTCTTTCT
Mrap Rev	TCCTGGCTCCTCTGTTGTCT
Nr5a1 Fw	TCCAGTGTCCACCCTTATCC
Nr5a1 Rev	CGTCGTACGAATAGTCCATGC
Osr1 Fw	ACTGATGAGCGACCTTACACCTG
Osr1 Rev	ACTTGTGAGTGTAGCGTCTTGTGGA
Paraxis Fw	GTGTAAGGACCGGAGGAGACAA
Paraxis Rev	GATGGCTAGATGGGTCCTTG
PAX2 Fw	CTGTTTCCAGCGCCTCTAAC
PAX2 Rev	GACGCTCAAAGACTCGATCC
PBX1 Fw	CAACTCAGTGGAGCATTCCGAC
PBX1 Rev	GGCTTTGCTCTCGAAGGAGGTT
Pdx1 L	GAAATCCACCAAAGCTCACG
Pdx1 R	CGGGTTCCGCTGTGTAAG
Prrx1 Fw	GATCTCGCACGTCGGGTGAACC
Prrx1 Rev	GCCAGCATGGCTCGCTCATTC
Sdha Fw	TGTTCAGTTCCACCCCACA
Sdha Rev	TCTCCACGACACCCTTCTG
Sox17 Fw	GATGCGGGATACGCCAGTG
Sox17 Rev	CCACCACCTCGCCTTTCAC
Sox2 L	TCCAAAAACTAATCACAACAATCG
Sox2 R	GAAGTGCAATTGGGATGAAAA
Sox9 Fw	CACAAGAAAGACCACCCCGA
Sox9 Rev	GGACCCTGAGATTGCCCAGA
StAR L	TTGGGCATACTCAACAACCA
StAR R	ACTTCGTCCCCGTTCTCC
T Fw	TCCCGAGACCCAGTTCATAG
T Rev	TTCTTTGGCATCAAGGAAGG
WT1 Fw	AGTTCCCCAACCATTCCTTC
WT1 Rev	CAACTGTCAGTAGGGGTGTGG

Table of primers used in the construction and validation of *Mc2r-Gfp*:

Sequence	Utility
atatgcttcaATGGTGAGCAAGGGCGAG	Egfp+polyA_Fwd (assembly)
caggaaaaaaTAAGATACATTGATGAGTTTGGACAAACC	Egfp+polyA_Rev (assembly)
ATAAGATACATTGATGAGTTTGG	Fw for EGFP-polyA
GATAGAGCCCAGCAAAGAGAG	Fw for Mc2r left homology arm
tgctcaccatTTTTTTCCTGCTGGCCGTTAAG	Fw gfp-Mc2r (assembly)
TCACAGGTATCCACAGAAGAAC	Fw left homology arm Mc2r amplification
AGGGAACAGCGATGTGAAGG	Fw Mc2r left homology arm-gfp genotype
ACAGCCAGGAGGACAATCAAG	Fw Mc2r left homology arm-gfp genotype
GAGTGTGATAGGTTAGAGCGAGTG	Fw Mc2r right homology arm genotype
taccgggccccccctcgaggTCACAGGTATCCACAGAAG	Fw Mc2r_pblue-1 (assembly)
GACGTTGTGGCTGTTGTAGTTG	GFP Fw genotype
ACCGCATCGAGCTGAAGG	GFP Rev genotype
atatgcttcaTTACTTGTACAGCTCGTCCATG	gfp_Fwd for Mc2r atg insertion (assembly)
caggaaaaaaATGGTGAGCAAGGGCGAG	gfp_Rev for Mc2r atg insertion (assembly)
taccgggccccccctcgaggGATAGAGCCCAGCAAAGAG	left homology arm Mc2r_Fwd (assembly)
tgctcaccatTGAAGCATATTATCAATTCGTATG	left homology arm Mc2r_Rev (assembly)
CCAGACCCAGGCTTCTGAAG	offtarget|Arhgef3_chr14_F
GCTTCCATGCCTCAGGACAT	offtarget|Arhgef3_chr14_R
CACAGCCAGGAGGACAATCA	ontarget insertion exon_Mc2r_F
GTAAGTCAACGGCAAACACCA	ontarget insertion exon_Mc2r_R
GCAATGACAGACAGGCTGAA	outside left homology arm Mc2r
GCAATGACAGACAGGCTGAA	outside left homology arm Mc2r
CGACCTTCAAGTCCGAGAAC	outside right homology arm Mc2r
ACGACCTTCAAGTCCGAGAA	outside right homology arm Mc2r
GCCCTTGCTTTGAGACTTTG	outside right homology arm Mc2r
GATAGAGCCCAGCAAAGA	Rev for EGFP-polyA
ACTGGCACTCGCTCTAACC	ReV for Mc2r right homology arm
gtacaagtaaTGAAGCATATTATCAATTCGTATG	Rev gfp-Mc2r (assembly)
CCCGCTCCCATGCTAGG	Rev Mc2r left homology arm-gfp genotype
CGTATGAACACACCAATGACACC	Rev Mc2r left homology arm-gfp genotype
GAGAGGTCCTTCCTTGTTTCCTG	Rev Mc2r right homology arm-gfp genotype
tatcaagcttatcgataccgAGATTCCAATCACCCTGAG	Rev Mc2r_pblue-1 (assembly)
AGATTCCAATCACCCTGAGAG	Rev right homology arm Mc2r amplification
atgtatcttaTTTTTTCCTGCTGGCCGTTAAG	right homology arm Mc2r_Fwd (assembly)
tatcaagcttatcgataccgACTGGCACTCGCTCTAAC	right homology arm Mc2r_Rev (assembly)

For the creation of the plasmid NEBuilder® HiFi DNA Assembly protocol was used with NEBuilder HiFi DNA Assembly Master Mix (**Ref** E2621), pBluescript was used as backbone vector. (assembly) indicates primers which were used to create the overlapping arms for the assembling procedure of each part.

6. Supplementary figures

Genotype	Male right	Male left	Female right	Female left
Cre^{-} - *Bmp4*$^{flx/-}$	0.0025	0.0022	0.0047	0.0048
Cre^{+} - *Bmp4*$^{flx/-}$	0.0022	0.0025	0.0056	0.0057
Cre^{+} - *Bmp4*$^{flx/flx}$	0.0027	0.0024	0.0040	0.0038

Figure.s1. Weight of adrenals in grams from transgenic mice. Genotype is depicted in the left column.

a.

Treatments after A.IM. stage

BMP4	BMP4+ RA + FGF2	Activin A	Purmo
BMP4 + Activin A	BMP4+ RA + FGF2 + BMP2	Activin A+FGF2	Purmo+Forskolin
BMP4 + BMP2	BMP4+ RA + FGF2 + BMP2+ IGF1	Activin A+Froskolin	Purmo+XAV-939
BMP4 + BMP2 + IGF1	BMP4+ RA + FGF2 + BMP5	BMP7	Purmo+XAV-939 +Forskolin
BMP4 + BMP5	BMP4+ RA + FGF2 + BMP5+ IGF1	BMP7+IPW-2	SHH
BMP4 + BMP5+ IGF1	BMP4+ RA + FGF2 + BMP6	DMH1	SHH+BMP4
BMP4 + BMP6	BMP4+ RA + FGF2 + BMP6+ IGF1	FGF2+RA+FGF9	SHH+CHIR
BMP4 + BMP6+ IGF1	BMP4+ RA + FGF2 + BMP7	FGF9	SHH+CHIR+BMP4
BMP4 + BMP7	BMP4+ RA + FGF2 + BMP7+ IGF1	FGF9+IGF1+IGF2+CHIR+LDN	SHH+CHIR+BMP4+FGF2
BMP4 + BMP7+ IGF1	BMP4+ RA + FGF2 + DLL4	Forskolin	SHH+FGF2
BMP4 + FGF2 + BMP2	BMP4+ RA + FGF2+ CD437 + BMP5	Forskolin+CHIR	SHH+FGF2+BMP4
BMP4 + FGF2 + DLL4	BMP4+ RA + FGF2+ RSPO1	Forskolin+FGF9	SHH+FGF2+FGF9+BMP4
BMP4 + FGF9	BMP4+ RA + FGF2+ RSPO3	IGF1	SHH+FGF2+RA
BMP4 +IPW-2	BMP4+ RA + FGF2+ Wnt-3a	IGF2	SHH+FGF2+RA+BMP4
BMP4+ BMP5	BMP4+ RA + FGF2+AA	IGF2+IGF1	SHH+FGF2+RA+FGF9
BMP4+ CD437	BMP4+ RA + FGF2+BMP5	IWP-2	SHH+FGF9+BMP4
BMP4+ CD437+BMP5	BMP4+ RA + FGF2+CD437	RA	SHH+FGF9+BMP4+FGF2
BMP4+ DLL4	BMP4+ RA + FGF2+DLL1	RA + FGF2+SB431542	SHH+FGF9+BMP4+FGF2+RA
BMP4+ FGF1	BMP4+ RA + FGF2+FGF1	SB431542	SHH+RA
BMP4+ FGF8b	BMP4+ RA + FGF2+FGF8b	SB431542+Forskolin	SHH+Wnt-3a+FGF1
BMP4+ RA	BMP4+ RA + FGF2+FGF9		SHH+Wnt-3a+FGF1+FGF2
BMP4+ RSPO3			SHH+Wnt-3a+FGF1+FGF2+RA
BMP4+ Wnt-3a			
BMP4+BMP7+FGF9+CHIR+LDN			
BMP4+BMP7+FGF9+IGF1+IGF2+CHIR			
BMP4+BMP7+FGF9+IGF1+IGF2+CHIR+LDN			
BMP4+BMP7+FGF9+IGF1+IGF2+LDN			
BMP4+BMP7+IGF1+IGF2+CHIR+LDN			
BMP4+DLL1			
BMP4+FGF1+DLL4			
BMP4+FGF1+DLL4+FGF2			
BMP4+FGF1+DLL4+FGF2+RA			
BMP4+FGF9+CHIR			
BMP4+FGF9+CHIR+FGF2			
BMP4+FGF9+CHIR+FGF2+RA			

Legend at next page

b. **Treatments at aggregate stage**

BMP4+ RA + FGF2	FGF2
cAMP	FGF2+FGF9+BMP4
cAMP+FGF9	FGF2+RA
cAMP+RA	FGF2+RA+acth
RA	FGF2+RA+FGF9
RA + FGF2+CHIR	FGF2+RA+FGF9+acth
RA + FGF2+CHIR+BMP4	FGF2+SHH
RA + FGF2+FGF9	FGF2+SHH+BMP4
RA + FGF2+FGF9+BMP4	FGF2+SHH+FGF9
RA + FGF2+FGF9+BMP4	Forskolin
RA + FGF2+FGF9+BMP4+BMP2	Forskolin +FGF2
RA + FGF2+RSPO1	Forskolin +insulin+IGF1
RA + FGF2+SHH	insulin+IGF1
RA + FGF2+SHH+BMP4	SHH
RA + FGF2+SHH+FGF9	SHH+FGF2
RA+cAMP+FGF9	SHH+FGF2+RA
	SHH+FGF2+RA+acth
	SHH+FGF2+RA+FGF9
	SHH+FGF2+RA+FGF9+acth
	SHH+Forskolin
	SHH+Forskolin +FGF2
	SHH+Forskolin +insulin+IGF1
	SHH+insulin+IGF1

c. **Treatments at new ECM**

BMP4	FGF9
BMP4	FGF2
BMP4+CHIR	FGF2+Activin A
BMP4+FGF9	FGF2+FGF9
BMP4+Forskolin	FGF2+FGF9+DMH1
BMP4+Purmo	FGF9+RA
cAMP	Forskolin
cAMP+BMP4	Forskolin+CHIR
cAMP+BMP4+Purmo	Forskolin+RA
cAMP+Dorsomorphin	Forskolin+Sb431542
cAMP+FGF9	IWP-2
cAMP+FGF9+Purmo	Noggin
cAMP+Noggin	Purmo
cAMP+Purmo	Purmo
CHIR	Purmo+BMP4
CHIR+Purmo	Purmo+FGFGF9
CHIR+RA	Purmo+Forskolin
CHIR+Sb431542	RA
DMH1	RA+BMP4
Dorsomorphin	RA+FGF9
	RA+Sb431542
	Sb431542
	Sb431542
	Sb431542+BMP4
	Sb431542+FGF9
	Sb431542+Purmo
	XAV-939

Figure.s2. Tables of factors tested at the *in vitro* differentiation protocol, (**a**) continuation of treatment in the same well after the A.IM stage, (**b**) treatments done at aggregates and (**c**) when cells were introduced to new ECMs.

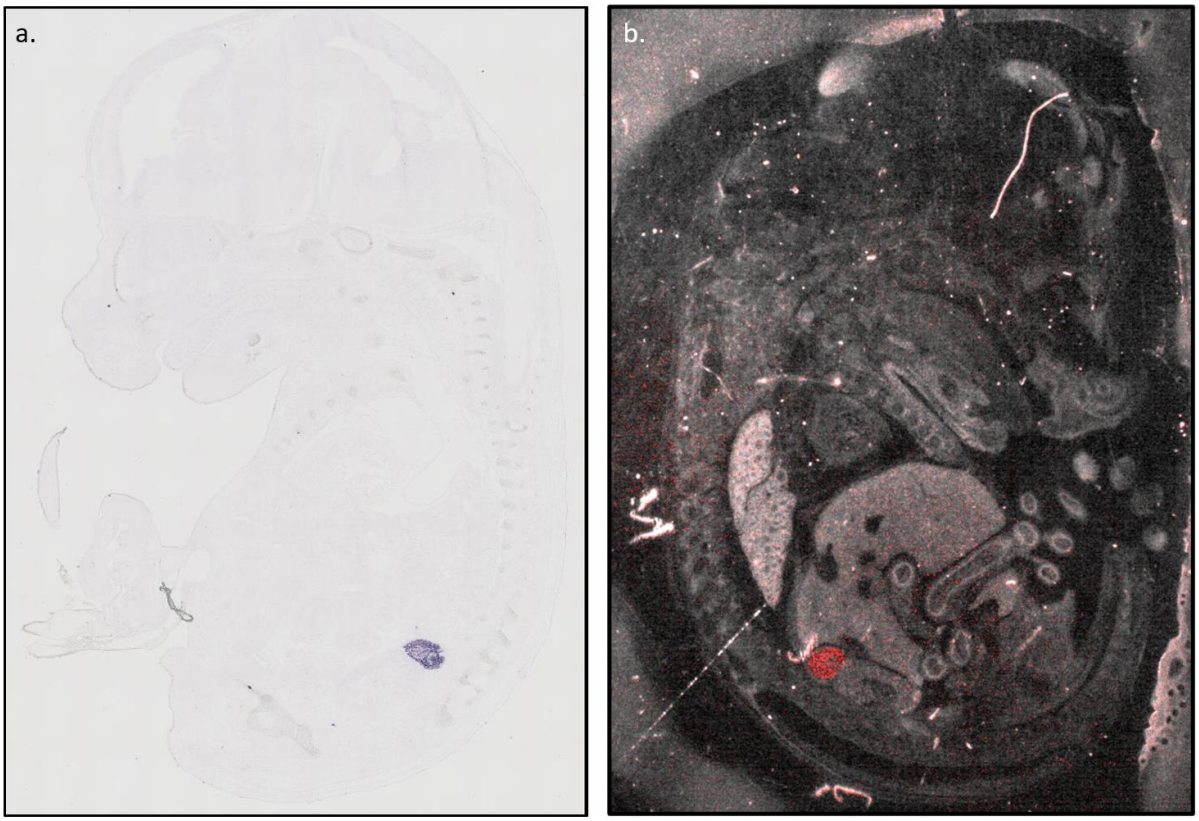


Figure.s3. RNA expression of *Mc2r* in e14.5 mice. (**a**) RNA *in situ* hybridization found on https://gp3.mpg.de/, (**b**) RNAscope for *Mc2r* (red).

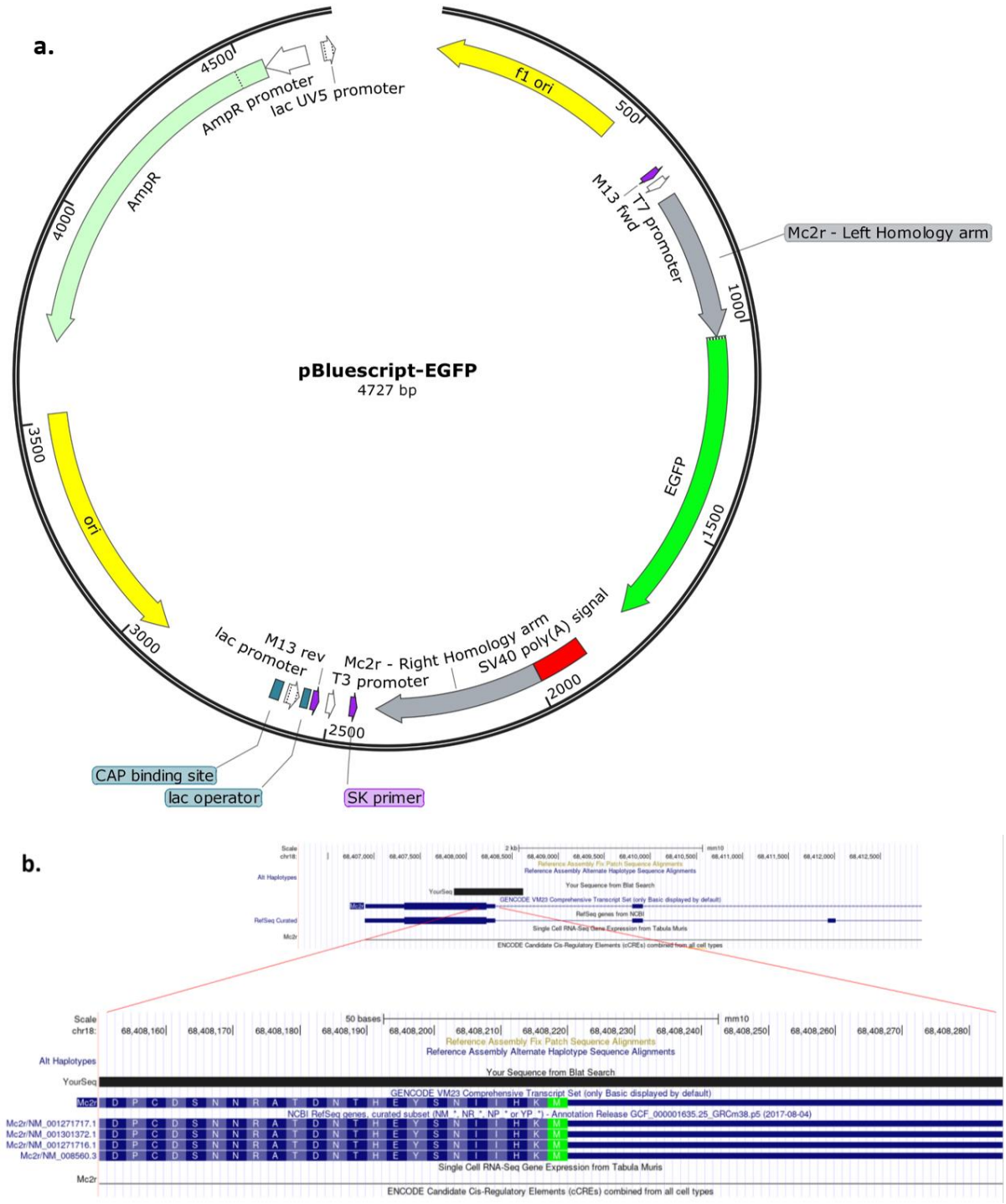


Figure.s4. Targeted insertion of *Egfp* in the *Mc2r* locus according to previous KO study (Chida et al., 2007). (**a**) *Egfp* inserted in pBluescript vector followed up by SV40 PolyA sequence. (**b**) Targeted locus of insertion in the *Mc2r* first Methionine.

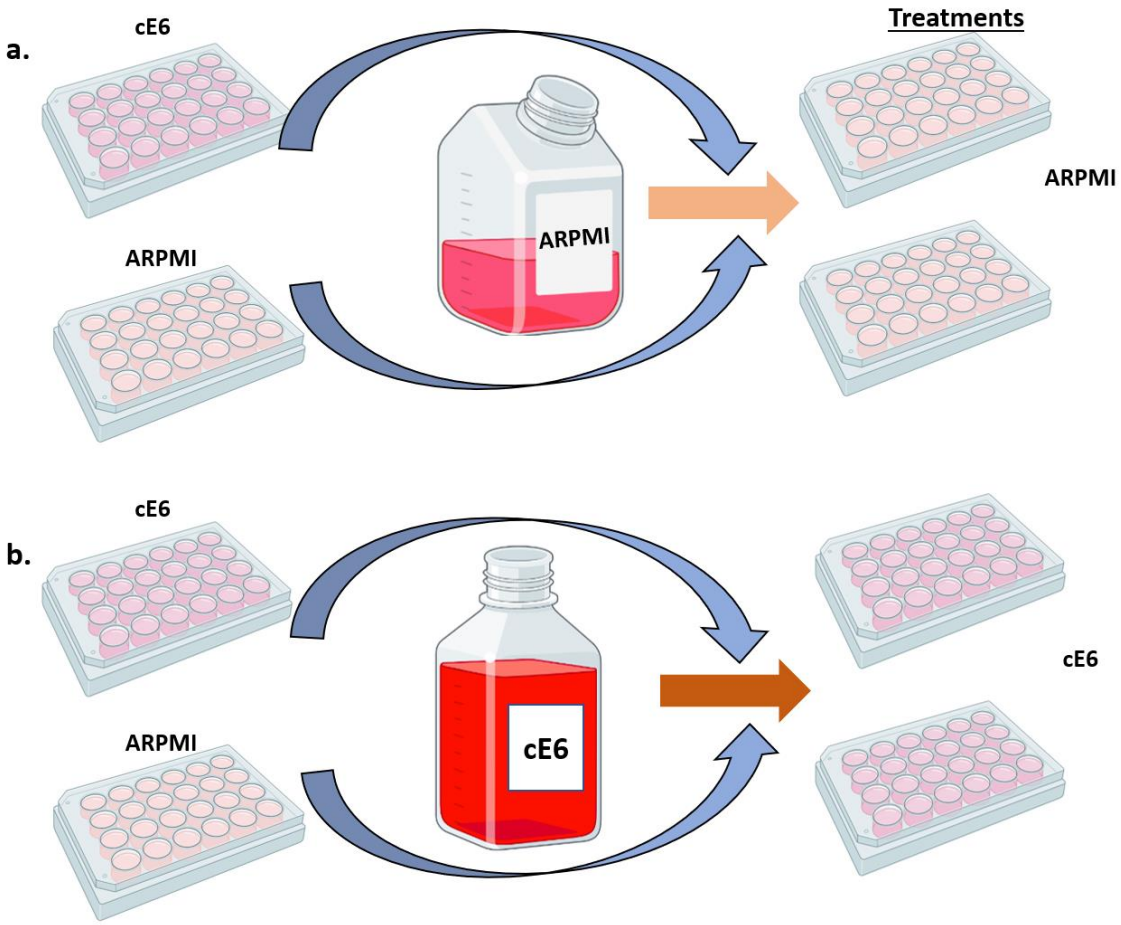


Figure.s5. Schematic of how the medium was alternated during treatments. After two days with M.CM in mentioned medium, the medium was replaced with either ARPMI (**a**) or cE6 (**b**) to carry one with the treatments.

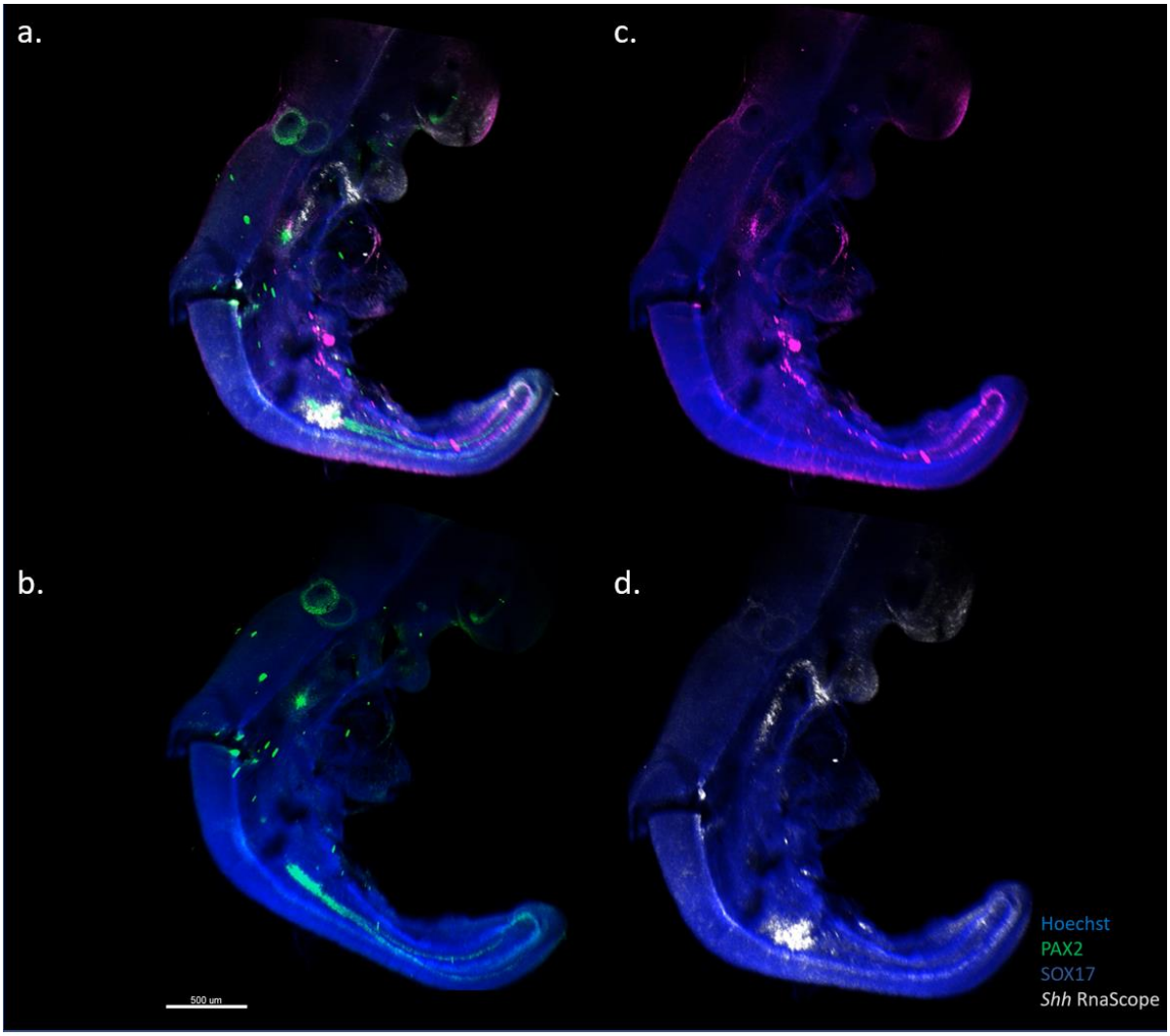


Figure.s6. Whole mount staining for PAX2 (**b**), SOX17 (**c**), combined with RNAscope for *Shh* (**d**) detection in e9.25 mouse. *Shh* is expressed at the notochord, head, hindgut, and foregut.

7. Abbreviations

2i two inhibitors (Chir and PD0325901)
A.IM anterior intermediate mesoderm
AM anterior mesoderm
AA Activin A
ACTH adrenocorticotropic hormone
AGP Adrenal-gonadal primordium
AP adrenal primordium
BAC Bacterial artificial chromosome
BMP Bone morphogenetic protein
BSA Bovine serum albumin
CAH Congenital adrenal hyperplasia
cAMP Cyclic adenosine monophosphate
CE coelomic epithelium
Chir Chir99021
CM conditioned medium
Col Collagen
CYP11A1 cytochrome P450 family 11 subfamily A member 1
CYP11B1 cytochrome P450 family 11 subfamily b polypeptide 1.
DAB2 Disabled 2
DZ definitive zone
E Embryonic day
ECM extracellular matrix
EpiSC Epiblast stem cell
FACS Fluorescence-activated cell sorting
FadE Fetal adrenal enhancer
FBS Fetal bovine serum
FGF Fibroblast growth factor
FN1 Fibronectin
FZ fetal zone
GFP Green fluorescent protein
GP gonadal primordium
h hour
Hepa Heparin
HH hedgehog
hIPSCs human induced pluripotent stem cells
IF immunofluorescence
IM intermediate mesoderm
KO knockout
Lam Laminin
LIF Leukaemia inhibitory factor
LPM Lateral plate mesoderm
M.CM mesodermal conditioned medium
MC2R Melanocortin receptor 2
ME medulla
MEF Mouse embryonic fibroblast
mESCs Mouse embryonic stem cells
min minutes
NBCS new-born calf serum
NDS normal donkey serum
NR5A1/ SF-1 nuclear receptor subfamily 5 group A member 1/ Steroidogenic factor 1
O/N overnight
PACAP Pituitary Adenylate Cyclase-Activating Polypeptide
PKA cAMP-dependent protein kinase
PS primitive streak
purmo Purmorphamine
RA Retinoic Acid
RT room temperature
RT-qPCR Reverse Transcription quantitative real-time polymerase chain reaction
SHH sonic hedgehog
StAR Steroidogenic acute regulatory protein
TH tyrosine hydroxylase.
TN Tenascin
ULA ultra-low attachment
VTN vitronectin
WT wild type
WT1 Wilm's tumour 1
zF zona fasciculata
zG zona glomerulosa
zR zona reticularis

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9. Acknowledgments

I should first and foremost thank Andreas for accepting me as his PhD student and to undertake this project among other candidates. We share the love for science and his enthusiasm was equally important for this project. I have always deeply appreciated his understanding over the difficulties of this project and PhD. He has been an exemplary scientific figure for me, and I hope I can learn more from him in the future. I would also like to thank him for spending the necessary time with me as an advisor, consequently understanding my scientific concerns and ideas, and in extend teaching me how to exist in a mature academic and scientific environment. In this aspect, I thank Dr. Jian Motamedi, Dr. Neirijnck and Dr. Massa for their critical comments over the project, which helped me to further develop as a scientist. I would like also to thank Dr. Vidal, Dr. Lyraki, Dr. Chaboissier, Dr. Perea and Msc. Weerasinghe, for their scientific insights through lab meeting and day to day discussions.

I would like to specifically thank Dr. Jian Motamedi for helping me initiate the *in vitro* project by guiding me through the techniques in the beginning, and sharing her protocol with me, which helped me create the work of this thesis in the long run. I thank Dr. Massa for teaching me the basics of CRISPR/Cas9 and aiding me in technical difficulties, in the beginning of my PhD. I would like also to highlight the contribution of Dr. Neirijnck through her assistance with the single cell RNA sequencing data; which although are not present in this manuscript, they have been helpful in guiding us in this differentiation experiment. Dr. Neirijnck also helped me in projects not present in this manuscript, but hopefully they will be helpful in the lab. Special thanks to Dr. Vidal for the scientific and psychological support that she provided to me and the whole lab. I have to say that I am also grateful for the arrival of the second PhD student in the lab, Msc. Weerasinghe, who provided me with scientific stimuli and discussion over my project and not only.

As I mentioned already, the single cells RNA seq analysis was performed by the lab of Prof. Nef and they had useful meetings with us and suggestions. Useful advises were given also by Dr. Dani as an internal advisor for my PhD. Dr. Swain was my external advisor, and she has provided us with scientific and experimental suggestions, and her knowledge was helpful for the project. She has been following the project since the beginning and I am very grateful for her time and input. Lastly, for the scientific part, I would like to put emphasis at the contribution of Dr. Van Obberghen on helping us understanding the basis of the ECM. She quickly got involved in the project in order to provide us with her scientific knowledge and her further input at the project. She and the PhD student REKAD Zeinab were willing to help us at any point with their knowledge and materials. Dr. Van Obberghen has also accepted the role of being the head of my viva jury, which greatly honours me.

I would like to thank all the people in and out of the academia who have supported me through the PhD, and thus supporting the finalisation of this thesis. Firstly, I would like to thank my parents for their continuous support with whatever mean they could; I could not have achieved this without them in the first place. I would also like to express my gratitude for my friends both in (Drs Avalos, Euthumiou, Friano, Garido and Tocco, and A. Vasiliadis) and out of academia, for their continuous support and advises in scientific and non-scientific related matters. Special thanks to my uncle and mentor Dr. I.C. Evagelopoulos for his counselling throughout my scientific career, and Dr. Kretsovali for her continuous support. I would like also to thank Petros for his friendship and continuous support, and Melissa (Dr. Sanabria) for her unconditional support in scientific and personal matters, which helps me to achieve my goals. Their support during these weird couple years with the pandemic had been crucial for me.

Finally, I would like to acknowledge Dr. Sekido, Dr. Steeblock (who also helped us with the steroidomics analysis) and Dr. Vainio for accepting to critically review my dissertation and to be part of PhD's viva jury. Of course this study would not be possible without the funding from IFCAH, thus we thank them for their work and trust on us.