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Establishment of Afamin-Wnt3a producer cells to generate serum-free growth medium for canine intestinal organoid culture.

Bachelor thesis

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1. Introduction

1.1. Intestinal Organoids

The epithelium of the intestine is known for its ability to form crypt-villus structures in order to absorb as many nutrients and electrolytes as possible. The intestinal lumen is covered by a monolayer of epithelial cells which also serves as a barrier against toxic substances and pathogens (Date and Sato, 2015). With a turnover rate of less than five days, the intestinal epithelium is the most vigorously self-renewing tissue of all mammalian tissues (Sato and Clevers, 2013). The gut consists of the small intestine, including duodenum, jejunum and ileum, and the colon (van der Flier and Clevers, 2009). The small intestine contains a large quantity of villi, which stick out into the lumen of the gut. At the base of the villi, invaginations, so called crypts of Lieberkühn, are located. In contrast to the small intestine (Figure 1A), the colon is folded into crypts but is missing villi (Figure 1B). At the bottom of the crypts of Lieberkühn reside intestinal stem cells (ISC) (Schuijers and Clevers, 2012) which Bjerknes and Cheng characterise as cells that are long-term self-renewing and multipotent (Bjerknes and Cheng, 2006). Approximately four to six stem cells are located in one crypt and are known for their production of rapidly proliferating cells, so called transit-amplifying (TA) cells (Sato et al., 2009). These progenitor cells divide four to five times before they differentiate along the crypt-villus axis into different secretory cells, such as enteroendocrine cells, goblet cells, Paneth cells and tuft cells, and absorptive enterocytes (Figure 1) (Sato and Clevers, 2013). Interestingly, in contrast to the small intestine, the colon is free from classically appearing Paneth cells (Date and Sato, 2015) but harbours Paneth-like cells according to single cell RNA-sequencing analyses (Wang et al., 2020).

Paneth cells are long-lasting cells which are located at the bottom of the crypts and have the function to produce antibacterial peptides to keep bacteria away from the epithelium of the crypts. Along the crypt-villus axis the following cells are scattered: goblet cells, enterocytes and enteroendocrine cells (Figure 1). These cells have functions like producing mucin and hormones, controlling digestive enzyme secretion, sensing neuroendocrine signals and absorbing nutrients (Date and Sato, 2015). Tuft cells are very rare and it is proposed that these cells play an important role regarding type 2 immunity to helminth infections (Gerbe et al., 2016).

Approximately, four days after the cells have finished their differentiation, they reach the tip of the villi and undergo anoikis, also known as detachment-induced apoptosis (Figure 1A). This means that the cells are sloughed off and shed into the lumen of the gut because of their loss of interaction with the extracellular matrix (ECM) (Beauséjour et al., 2013).

In 1974 Cheng and Leblond described crypt base columnar (CBC) cells for the first time which are intermingled between the Paneth cells at the bottom of the crypts and were thought to be the origin of all differentiated intestinal cells (Cheng and Leblond, 1974). After this discovery the progress to identify these cells slowed down because of missing markers (Date and Sato, 2015). However, 2007 scientists discovered a marker gene, which helps to identify stem cells not only in the small intestine and colon but also in other adult tissues and cancers. The marker was the leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) also called GPR49. The *LGR5* gene is a Wnt target gene and in the small intestine and colon only CBC cells are expressing this gene suggesting that it is a stem cell marker gene (Barker et al., 2007). A few years later, it was shown that small intestinal LGR5^{+} stem cells cultured in a specialised medium and extracellular matrix can develop similar structures *in vitro* without needing a mesenchymal niche (Sato et al., 2009). This finding was of utmost importance for the cultivation of organoids and for the understanding of the pathways regulating the intestinal stemness of three-dimensional (3D) cell culture. The term “stemness” comprises the ability for lifelong self-renewal and the multipotent differentiation potential (Date and Sato, 2015).

1946 Smith and Cochrane used the term “organoid” as a synonym for a teratoma (Smith and Cochrane, 1946). Since the 1960s the term was used for an organotypic cell collection that had their origin in a stem cell and had the ability to self-organise into an organlike tissue (Lancaster and Knoblich, 2014). Some years later Kretschmar and Clevers refined the definition from organoids as

> *“three-dimensional (3D) in vitro grown structures derived from PSCs* or ASCs^{+} that self-organize into a near-native microanatomy with organ-specific differentiated cell types and tissue compartmentalization.”* (Kretzschmar and Clevers, 2016).

**PSCs (pluripotent stem cells), $^{+}$ASCs (adult stem cells)*

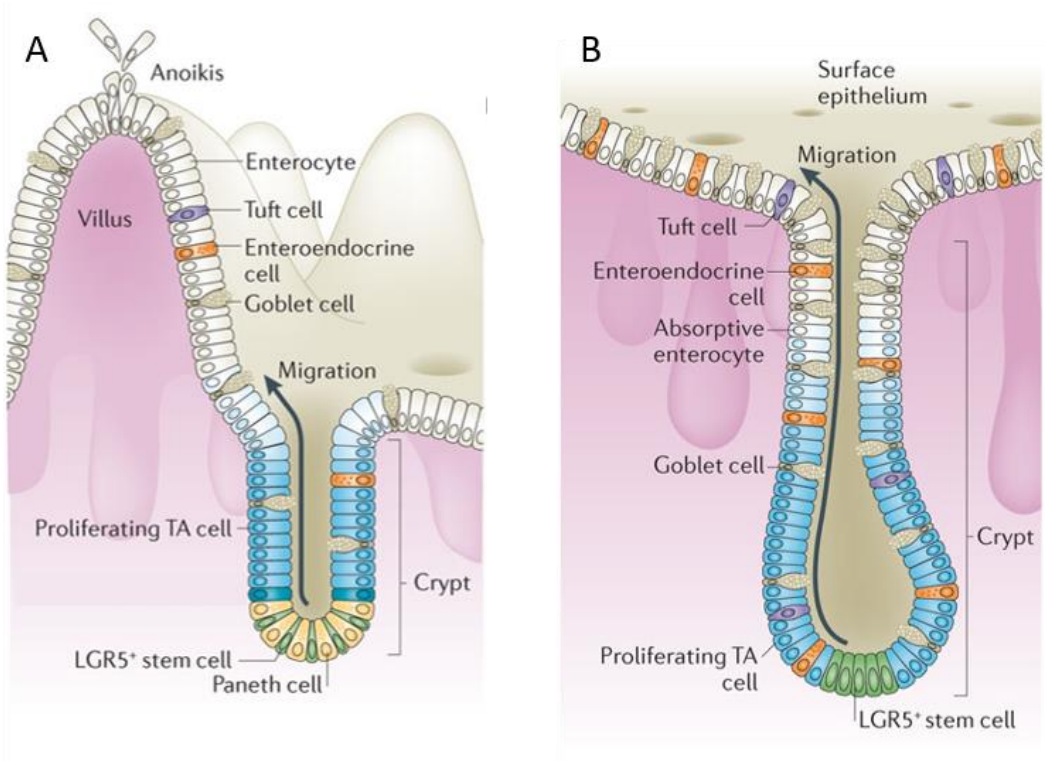

Figure 1: *Structure and cell types of the small intestine (A) and colon (B). Adapted from (Barker, 2014)*

But how is that possible?

For a long time, it was very difficult to increase the number of adult intestinal epithelial cells without changing their DNA. 1992 a scientist triumphed by adhering crypt epithelial cells to a culture dish which was coated with collagen type I. The cells could be cultured for 1-2 weeks but the survival of these cells depended on the interaction with subepithelial fibroblasts. Without these contaminating cells, the epithelial cells were unable to grow (Evans et al., 1992). In 2009 an air-liquid interface was established by Ootani and colleagues for long-term culture of organoids from neonatal intestinal mucosa and mesenchyme (Ootani et al., 2009). But these systems have one problem: both need the co-culturing of mesenchymal fibroblast to expand, pointing out that the cells of the mesenchyme serve as a part of the ISC niche (Date and Sato, 2015). A niche represents a stem cell microenvironment which regulates cell fate (van der Flier and Clevers, 2009). However, for some questions the co-cultivation of mesenchymal fibroblasts is a major disadvantage and therefore a model lacking mesenchymal cells would help to investigate epithelial intrinsic functions or defects and would reduce potential experimental noise, which is caused by the mesenchyme (Mithal et al., 2020).

However, as already mentioned, organoids can be cultured for a long time from a single $LGR5^+$ stem cell without needing a mesenchymal niche, suggesting the existence of other cells serving as a stem cell niche (Sato et al., 2009). Sato and colleagues claim that Paneth cells are an essential part of the stem cell niche and that $LGR5^+$ stem cells have to be next to them in order to be maintained. The factors provided by the Paneth cells are Wnt, EGF, TGFα and Notch ligand Dll4 (Sato et al., 2011b).

In order to establish a 3D mini-gut culture with organoids that are characterised by crypts and villi and have the ability for long-term self-renewal, it is very important to culture the isolated stem cells in an unique environment, which contains the niche factors, extracellular matrix (ECM), Noggin and R-spondin (Date and Sato, 2015).

The interaction between the epithelium and the basal lamina is also very important regarding the survival of epithelial cells. As mentioned previously, a loss of the interaction inhibits integrin signalling and the cell induces anoikis. In order to prevent this detachment-induced apoptosis *in vitro*, it is essential to embed organoids in ECM. ECM serves as a replacement for the basal lamina (Date and Sato, 2015) and it consists mainly out of laminins, elastins, collagens and fibronectins (Alberts et al., 2015).

For the long-term cultivation of canine intestinal organoids, the research group of Prof. Iwan Burgener established a refined medium which supports long-term expansion of organoids but simultaneously allows differentiation into enterocytes, goblet cells and enteroendocrine cells. Based on the niche-inspired culture condition of Fujii and colleagues (Fujii et al., 2018) the basal medium consists of Advanced DMEM/F12 supplemented with GlutaMAX, HEPES, B27 and N-acetylcysteine, enriched with Noggin, R-spondin-1, Wnt3a, gastrin, A83-01 (Alk4/5/7 inhibitor), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1) and fibroblast growth factor-2 (FGF-2). For the first two days after the isolation also epidermal growth factor (EGF) and Y-27632 (ROCK inhibitor) were added to the refined medium (Kramer et al., 2020).

EGF, Noggin and gastrin need to be added to regulate the gut mucosal growth, but they are also crucial for the proliferation of intestinal epithelial cells. To control self-renewal, differentiation and proliferation of stem cells the addition of Wnt3a is fundamental. Regarding the canonical Wnt signalling pathway, which will be explained in detail later in this thesis, also R-spondin1 and Noggin are of utmost importance because R-spondin1 supports Wnt3a and Noggin inhibits bone morphogenic protein 4 (BMP4) (Fair et al., 2018) which lead to a greater

number of ISCs (Davis et al., 2015). IGF-1 is important for the hyperplasia of crypts whereas FGF-2 plays a major role in the regeneration of injured tissue, which supports the fact that FGF-2 is an essential niche factor (Fujii et al., 2018). A83-01 is a small molecule inhibitor of receptor-like kinases (ALK4/5/7) and increases the number of cells which originate from one single $LGR5^+$ stem cell (Sato et al., 2011a). EGF and HGF are not necessary for the cultivation but each increase the growth of organoids (Yui et al., 2012). In order to prevent anoikis in the first two days and to enhance the survival of freshly isolated organoids the supplementation of Y-27632 is essential. Chandra and colleagues also claim that the addition of Y-27632 leads to a better colony forming efficiency of organoids (Chandra et al., 2019).

Only if the responsible factors are known for the correct reflection of the intestine *in vivo* it is possible to develop organoids that mirror the correct physiology and the heterogeneity of the intestinal epithelium (Blutt et al., 2019). That is exactly the reason why organoids are the perfect model for many applications ranging from basic research to personalised medicine (Artegiani and Clevers, 2018).

First, organoids could be used as a model to study infectious diseases and the interaction of host and pathogen (Artegiani and Clevers, 2018). For instance, in an experiment Helicobacter pylori were injected into gastric organoids to see the primary reaction of the epithelium to the infection using microarray analysis and to notice that the gastric gland lineages have the strongest inflammatory reaction (Bartfeld et al., 2015).

Second, organoids serve as a model for genetic diseases (Artegiani and Clevers, 2018). Especially in combination with clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9 (Cas9) technology organoids are the perfect tool to make findings regarding mutational effects that occur in embryonic stages and could even be fatal. For example, organoids derived from patients with atresia, showed that the mutation responsible for this disease leads to an inadequate polarity of the intestinal epithelium and that by inhibiting Rho kinase signalling normal polarity could be regained (Bigorgne et al., 2014).

Third, Organoids could be used for tumour modelling and to establish a living biobank (Artegiani and Clevers, 2018). Organoids developed from tumour tissue are called “tumouroids”. These tumouroids can be collected from many cancer types from many different patients and are then called a living biobank. Biobanks include many tumouroid lines which mirror the pathological variety of cancer and can therefore be used for functional tests,

genotype-phenotype correlation analysis or to test drug response (Artegiani and Clevers, 2018). Wild-type organoids can also be used to mutate genes, which are under suspicion to cause cancer. In this way scientists discovered that mutations of the genes *APC*, *TP53*, *KRAS* and *SMAD4* lead to colorectal cancer development *in vitro* (Drost et al., 2015).

Fourth, organoids can be used to repair genetic defects (Artegiani and Clevers, 2018). As already mentioned organoids have the ability to multiply without being genomically instable (Sato et al., 2011a). This ability paired with CRISPR/Cas9 technology offers the possibility to repair the mutation causing a genetic disease by genome editing. In the year 2013 organoids were cultivated from stem cells derived from patients with cystic fibrosis. The mutation ΔF508 in the *CFTR* gene was repaired by using a Cas9-mediated homologous recombination with a wild-type *CFTR* gene (Schwank et al., 2013).

Fifth, organoids are a model to study cell development and gene function. Organoids can be used to answer basic research questions such as studying the development of genes and their function (Artegiani and Clevers, 2018). For instance, removing deep crypt secretory cells from the colon leads to the loss of $LGR5^+$ stem cells, underlining that these cells have a similar niche function as Paneth cells in the small intestine (Sasaki et al., 2016). 3D cell culture is also very helpful to understand the role of signalling pathways which may be important for the stemness of organoids. For example, by removing Notch, Wnt and EGF-receptor or by inhibiting mitogen-activated protein kinase (MAPK) signalling, the cells of organoids start to differentiate into enteroendocrine cells (Basak et al., 2017).

Even though there are a lot of astonishing applications of organoids many of them are just shown at a proof-of-principle level and are rarely used (Artegiani and Clevers, 2018). Therefore, there are a lot of future challenges which have to be overcome so that organoids can develop their full potential (Fair et al., 2018). One challenge will be to reduce or replace ECM. ECM is a badly described protein cocktail (Shuhendler et al., 2013) which adds inconsistence to the cell culture and may therefore influence the mirrored physiology (Hughes et al., 2010). This cocktail is obtained from a murine Engelbreth-Holm-Swam (EHS) tumour (Kleinman et al., 1986) and is therefore animal-derived which generates an abnormal environment for organoids derived from other species (Almeqdadi et al., 2019). Also, in regenerative medicine the clinical application of organoids assumes chemically defined, animal origin-free supplements. However, ECM is not the only animal-derived product which is

necessary for the cultivation of intestinal organoids. In order to maintain the stability of Wnt ligands in culture medium, foetal bovine serum (FBS) is essential (Date and Sato, 2015). For medical legislation it is necessary to establish cell culture conditions which do not require animal products if organoids should be used for direct transplantations in future (Kretzschmar and Clevers, 2016). The cultivation of organoids is also missing other elements of the *in vivo* organ, such as neural, immune and mesenchymal cells which influence the characteristics of the intestine (Fair et al., 2018). Organoid culture also entails practical challenges. For example, embedding organoids in ECM, so they can grow in 3D, make manageable manipulation and gene editing attempts awfully complex (Fair et al., 2018). Also, delivering organisms, toxins or drugs into the lumen of organoids is complicated due to the fact that the apical surface is facing towards the intestinal lumen. One solution would be to use microinjection but this method is inefficient and therefore alternatives would be better suited (Fair et al., 2018). Despite the fact that 3D cell culture entails many challenges and limitations, overcoming these challenges will help to develop an *in vitro* model which can not only help to reduce animal experiments but can also be used for disease modelling, drug discovery and for personalised medicine.

As already mentioned, long-term cultivation of canine intestinal organoids for *in vitro* modelling relies medium supplemented with special niche factors. One of the most important factors is Wnt3a, which activates the canonical Wnt signalling pathway, which is crucial for the maintenance of the stem cell niche and various processes in embryonic development.

1.2. Wnt Signalling

Wnt signalling is a highly conserved cell-communication pathway which is activated when a Wnt molecule binds on the cell surface from another cell. Due to this interaction different intracellular biochemical events take place in the receiving cell (Söderholm and Cantù, 2021). This cascade can be divided into three types. The first one is called canonical Wnt signalling pathway and will be described in more detail below. The other two are noncanonical planar cell polarity pathway and noncanonical Wnt/calcium pathway (Clevers, 2006). The latter ones will not be topic of this thesis.

1982 the gene *INT-1* was discovered by Nusse and Varmus as a proviral integration site for the murine retrovirus mouse mammary tumour virus (MMTV) which is known for its ability to cause breast tumours (Nusse and Varmus, 1982). First, it was thought that this gene is a novel proto-oncogene but after elucidating the structure and the sequence Rijsewijk and his

colleagues noticed that it is homologous to the fly gene *Wingless (WG)* (Rijsewijk et al., 1987). *Wingless* was first described by Sharma and Chopra by showing that a weak allelic mutation in *Drosophila melanogaster* leads to the loss of wings and/or halters (Sharma and Chopra, 1976). In 1980 Nüsslein-Vollhard and Wieschaus showed that mutations in genes that are essential for the development of the larva in *Drosophila* lead to an unusual number of segments and change the polarity. Hence, they named these genes “segment polarity genes”. Among these are *Wingless* and *Arrow* (Nüsslein-Volhard and Wieschaus, 1980). To prevent confusion researchers decided to combine *Wingless* and *INT-1* to *WNT-1* (wingless-type MMTV integration site family, member 1) (Nusse et al., 1991).

Until now mammals contain a family of 19 Wnt ligands (Nie et al., 2020). These proteins are characterised by their highly conserved distribution of cysteines (Wodarz and Nusse, 1998) and their hydrophobic nature (Kikuchi et al., 2007). This insolubility of Wnt in aqueous solutions is the result of a palmitoylation. This modification takes place in the endoplasmic reticulum (ER) and is found on one of the conserved cysteines. It was shown that by removing this palmitic acid the Wnt ligands are no longer hydrophobic and also inactive, suggesting that the palmitoylation is crucial for the signalling pathway (Willert et al., 2003).

The canonical Wnt pathway is also called Wnt/β-catenin pathway because in addition to Wnt ligands, β-catenin is crucial for this cascade and therefore important for stem cell maintenance, embryonic development and adult homeostasis (Logan and Nusse, 2004).

β-catenin is regulated by Wnt ligands. Hence, if the Wnt signal is absent the stability of β-catenin is controlled by a multiprotein destruction complex (Figure 2) (MacDonald et al., 2009). Axin is the scaffolding protein of this complex and uses domains to interact with kinases, phosphatases, β-catenin and the adenomatous polyposis coli (APC) protein (Kimelman and Xu, 2006). The two kinases are called casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK-3). CK1 phosphorylates β-catenin at threonine 41 and serine 45 and GSK-3 phosphorylates at serine 33 and 37. The latter modifications are crucial for the destruction of β-catenin because it is the binding site for the E3 ubiquitin ligase β-Trep which ubiquitinates β-catenin leading to its degradation through the proteasome (Figure 2) (Kimelman and Xu, 2006). This proteolytic system is important because it keeps the cytosolic levels of β-catenin low and inhibits the translocation to the nucleus (van Kappel and Maurice, 2017). Interestingly, APC and Axin can bind to the same region of β-catenin, leading to the proposal that APC may stabilise the Axin-β-catenin complex (Ha et al., 2004). Other research groups claim, that the

same binding site is essential for removing the phosphorylated β-catenin from Axin for degradation and setting Axin free for further phosphorylation of β-catenin (Stamos and Weis, 2013). But it is unclear if β-catenin has to dissociate from the complex to be degraded (Hart et al., 1999).

Frizzled (Fzd) proteins are seven-pass transmembrane receptors with a cysteine-rich domain (CRD) serving as the direct binding site for Wnt ligands (Bhanot et al., 1996). As soon as the Wnt protein binds to its primary receptor, the canonical Wnt pathway gets activated (Figure 2) (Clevers, 2006).However, the interaction between Wnt and Frizzled is not enough to get the pathway started. Therefore, a single-pass transmembrane molecule of the LRP family is necessary. In *Drosophila* this protein is called Arrow (Wehrli et al., 2000) and in vertebrates Leucine-rich repeat-containing G-protein coupled receptor 5 and 6 (LRP5 and -6) (Pinson et al., 2000). Frizzled can directly interact with Dishevelled (Dvl), a cytoplasmic scaffolding protein, which binds to Fzd's C-terminal Lys-Thr-X-X-X-Trp motif (Umbhauer et al., 2000) and gets phosphorylated (Yanagawa et al., 1995). Until now it is unclear how the signal from the receptor is forwarded to Dishevelled (Anthony et al., 2020). Some scientists made the hypothesis that G-protein signalling is involved as they share the same seven-pass-transmembrane structure (Katanaev et al., 2005) but the underlying mechanisms remain unclear. Equivalent to Fzd also LRP5/6 interacts with a protein that is crucial for the Wnt signalling (Logan and Nusse, 2004). LRP5/6 has a cytoplasmatic tail which consists of Pro-Pro-Pro-(Ser/Trp)-Pro motifs that are modified with a phosphoryl group after the binding of Wnt ligand to the Fzd/LRP5/6 receptor (Tamai et al., 2004). Due to this modification LRP5/6 offers a docking site for Axin (Mao et al., 2001). The sequences of Dvl and Axin are both coding for the Dishevelled-Axin (DIX) domain. Therefore via this domain the proteins can interact with each other and heterodimerize, leading to the disintegration of the multiprotein destruction complex and to stabilisation of β-catenin (Itoh et al., 2000). There is evidence, that not the elimination of the destruction complex is responsible for the release of β-catenin but rather the need of Axin at the receptor complex which reduces the available number of Axin molecules in the cytoplasm (Lee et al., 2003).

After disintegration the multiprotein destruction complex, β-catenin accumulates in the cytosol and translocates to the nucleus (Tolwinski and Wieschaus, 2004). In the nucleus, β-catenin interacts with the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor family (Figure 2) (Behrens et al., 1996).This family contains four transcription factors (TCF7, LEF1,

TCF7L1 and TCF7L2) which among other things regulate the gene expression in immune cells and neural crest during embryogenesis (Brantjes et al., 2001). TCF factors bind the DNA in a unique way. They bind the recognition motif AGATCAAAGG in the minor groove of the DNA helix, hence inducing a 90° bend (van de Wetering et al., 1997). Without any Wnt ligands, TCFs act as transcriptional repressors by interacting with Groucho proteins (Cavallo et al., 1998). As soon as β-catenin arrives in the nucleus TCFs are no longer transcriptional repressors because β-catenin displaces Groucho from TCF/LEF and recruits the histone acetyltransferase CBP/p300 (cyclic AMP response element-binding protein) (Hecht et al., 2000), a component of the chromatin remodelling complex Brg-1 (Barker et al., 2001) leading to transcription initiation and elongation (Mosimann et al., 2006). Many target genes are associated with Wnt signalling and by using mutant analysis the large range of biological processes that are controlled by Wnt was shown. For example, Wingless plays an important role in Drosophila regarding embryonic development and larval patterning (Cadigan and Nusse, 1997). In vertebrates mutations can for instance lead to embryonic lethality, kidney and limb defects and central nervous system (CNS) abnormalities. These results indicate that the gene regulation is cell-type specific (Logan and Nusse, 2004).

There are also other proteins which activate the canonical Wnt signalling pathway. For instance, R-spondin1 can interact with LRP6 and Frizzled (Nam et al., 2006) and hence stabilise β-catenin which starts the expression of genes that promote the proliferation of intestinal crypt cells (Kim et al., 2005). Another ligand is the cysteine-knot protein Norrin. Norrin can bind to Frizzled-4 and LRP5/6 and activates the pathway (Xu et al., 2004). It plays a central role in the vascular development of eyes and ears (Gal et al., 1996).

Wnt3a is the Wnt ligand which is secreted by Paneth cells and therefore a niche signal. Hence it plays a major role regarding stem cell maintenance (Sato et al., 2011b). In contrast to other Wnt proteins, Wnt3a undergoes two palmitoylations, which makes it even more hydrophobic (Hausmann et al., 2007). The first one is attached to cysteine 77, which is necessary for the control of extracellular transport and the second to serine 209, which is crucial for intracellular trafficking during secretion (Takada et al., 2006). Due to these modifications Wnt3a is insoluble in aqueous solutions (Willert et al., 2003). However, as already mentioned, canine intestinal organoids need medium supplemented with Wnt3a and therefore, it is necessary to bring it into a water-soluble form. This is achieved by cultivating Wnt3a expressing L-cells (murine fibroblastic cells) in DMEM containing 10 % foetal bovine serum (FBS) (Willert, 2008). FBS

contains the protein Afamin which forms a complex with Wnt3a. This complex is then water-soluble (Mihara et al., 2016). After one week of cultivating L-Wnt3a cells without changing the medium, the medium can be harvested and is then called conditioned medium (c.m.). The FBS-containing Wnt3a conditioned medium serves as the basis for the organoid cultivation medium. However, the use of organoids in the clinical field of regenerative medicine requires culture with animal-origin free substances. To achieve this goal, the development of FBS-free culture conditions is required.

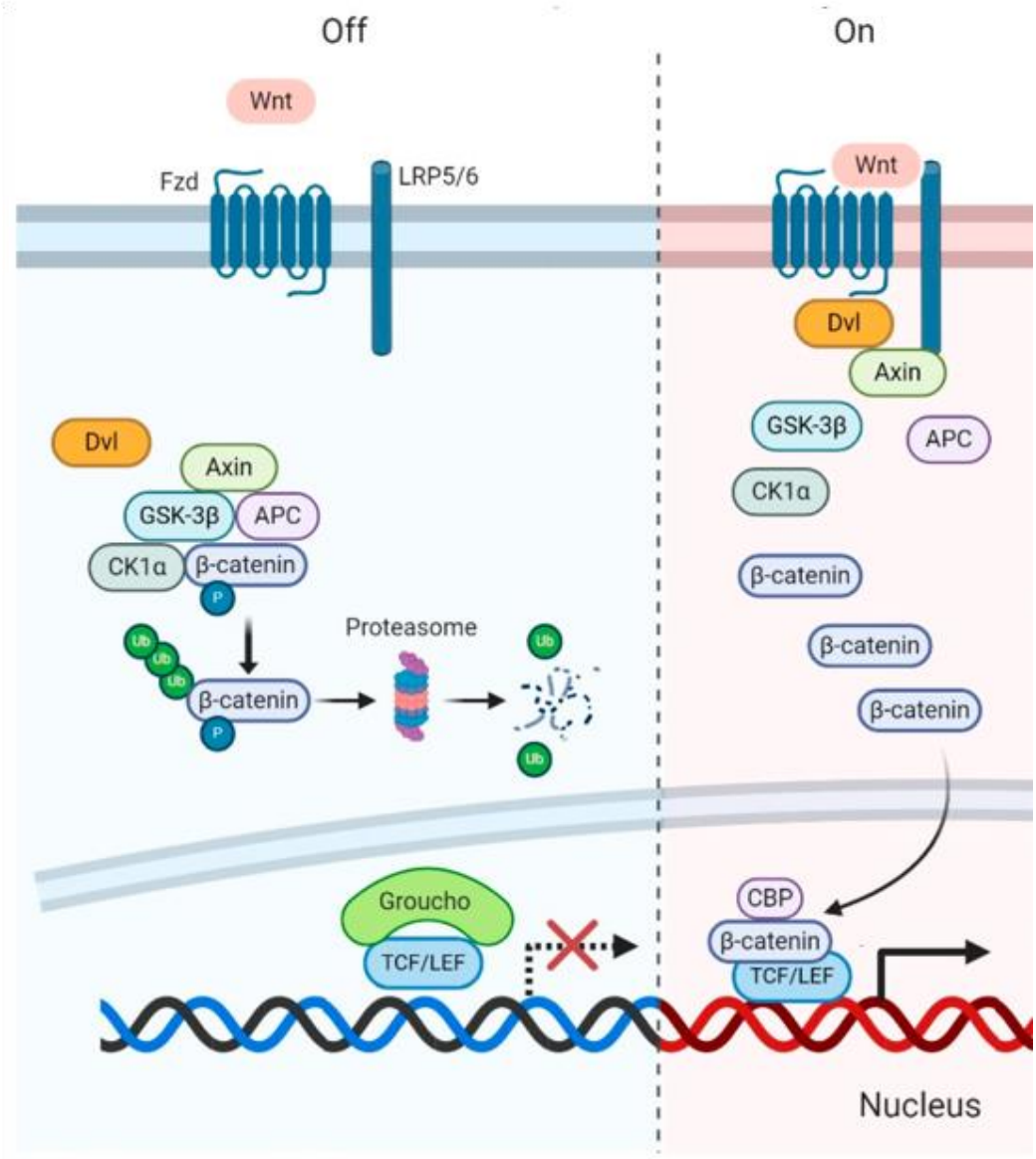

Figure 2: *Canonical Wnt-β-catenin pathway. Adapted from (Ruan et al., 2020)*

1.3. Reasons why to avoid the Usage of Foetal Bovine Serum (FBS)

FBS is obtained by slaughtering a pregnant cow and its foetus at any time during the last two thirds of pregnancy to collect the foetal blood (Jochems et al., 2002). In the 1950s Puck discovered that FBS stimulates cellular growth (Puck et al., 1958). Later on it was determined that FBS contains hormones, transport proteins, vitamins, trace elements, spreading and growth factors which are crucial for cell proliferation and maintenance (Brunner et al., 2010). Since then FBS was established as an inevitable supplement in cell culture medium (van der Valk et al., 2018). Comparing to other sera FBS is especially often used because of its small amount of complement factors and immunoglobulins (Gstraunthaler and Lindl, 2013). In the year 2012 it was estimated that worldwide 800,000 litres of FBS are used annually. This corresponds to 2,000,000 foetuses (van der Valk et al., 2018).

The necessity to kill animals is not the only disadvantage of FBS. The major downside of using FBS for organoid culture is that in regenerative medicine the clinical application of organoids assumes chemically defined, animal origin-free media and supplements. Otherwise it will not be possible to transplant the patient-derived organoids back into the patient because of medical legislation (Kretzschmar and Clevers, 2016). Also, the undefined composition, the batch-to-batch variation and the high possibility of contamination are serious obstacles (Yao and Asayama, 2017). Therefore, the replacement of animal-derived products in intestinal organoid culture is essential.

However, a critical point for the production of animal-origin free conditioned media is the insolubility of Wnt proteins in aqueous solutions and its purification due to their hydrophobic properties (Naschberger et al., 2017).

In 2008 it was discovered that the use of the detergent CHAPS at 1 % helps to make Wnt3a soluble. Hence it was possible to purify many Wnt ligands which could later be used for different experiments (Willert, 2008). However, this method entailed some problems like the purified Wnts contained CHAPS and they were unsuitable for detergent-sensitive cell-based assays. The biggest challenge was that the Wnts had to be transported into lipid vesicles, if they had to be incubated at 37 °C to remain biologically active (Dhamdhere et al., 2014). 2016 Mihara and colleagues discovered that the protein in FBS which is responsible for the solubility of Wnt3a is the serum glycoprotein Afamin (Mihara et al., 2016). Combining Afamin and Wnt3a in a 1:1 ratio makes a complex which is not only water soluble but also biologically active

(Mihara et al., 2016). These results suggest that Afamin can serve as a carrier and deliver Wnt proteins to its receptors on the surface of neighbouring cells. The Afamin-Wnt3a complex can even be stored at 4 °C without losing its activity (Mihara et al., 2016).

Based on this knowledge, the generation of Afamin-Wnt3a producing cells to obtain FBS-free growth medium for the cultivation of canine intestinal organoids was the focus of this thesis. For this purpose, we used a piggyBac vector for expression of Afamin and Wnt3a (Figure 3) and the Super piggyBac transposase expression vector for the transfection of human embryonic kidney 293 cells (HEK293T) and L-Wnt3a cells. These cell lines were chosen to investigate which of these cells are better suited for the production of serum-free conditioned media containing Wnt3a. HEK293T cells are known for their ability to be easily transfected (“293T | ATCC,” n.d.). L-Wnt3a cells were kindly provided by Hans Clevers (Hubrecht Institute, Utrecht, Netherlands). After transfection and selection with Puromycin we verified the genomic integration via PCR and harvested conditioned media from both cell lines. Subsequently, the Wnt3a functionality, viability, apoptosis and necrosis on organoid culture were checked. To verify the biological activity of Afamin-Wnt3a on intestinal cells, we also cultivated canine colon organoids for 28 days in different conditioned media productions and documented their growth every second day.

2. Material and Methods

2.1. Plasmid Amplification and Isolation

For the preparation of the lysogeny broth (LB) medium five tablets of LB Broth with agar (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in MilliQ-water and autoclaved (Fedegari Group, Pavia, Italy). For the LB agar plates the LB agar was microwaved until the agar was completely dissolved and homogenised. 10 ml of LB agar were mixed with 10 µl of Ampicillin (Sigma-Aldrich) and poured in 90 mm Petri Dishes (Semandeni, Ostermundigen, Switzerland). After cooling down the plates were sealed with parafilm and stored inverted at four degrees.

The Afamin and Wnt3a plasmid BII-CMV-AfmW3A (Addgene, Watertown, MA, USA, seen in Figure 3) was obtained as a bacterial stab. The growing bacteria were picked with a sterile pipette tip and transferred to 5 ml of LB medium without antibiotics and incubated on a shaking platform at 37 °C overnight. On the next day, 100 µl of the suspension were spread on LB agar plates and incubated at 37 °C overnight.

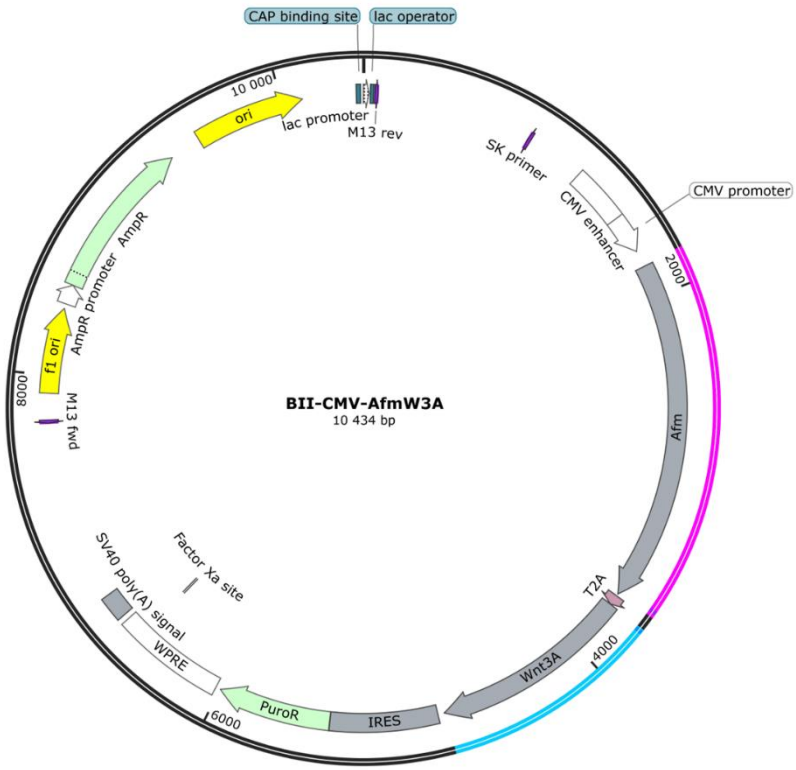

Figure 3: *BII-CMV-AfmW3A plasmid. Adapted from ("Addgene: BII-CMV-AfmW3A," n.d.)*

For large-scale production of plasmid, a single colony was picked from the agar plate with a pipette tip and dropped into 50 ml of LB medium and incubated overnight at 37 °C.

In order to prepare a glycerol stock, 2 ml of LB medium were mixed with one colony of bacteria and left at 37 °C in a shaker overnight. The next day 500 µl of 50 % glycerol (AppliChem, Chicago, IL, USA) and 500 µl of the bacteria suspension were mixed to obtain a final concentration of 25 % glycerol and stored at -80 °C.

The BII-CMV-AfmW3A plasmid was purified with the Pure Yield Plasmid Midiprep System (Promega, Madison, WI, USA) according to the instructions of the manufacturer. In brief, the selected bacteria grown in 50 ml of LB medium were pelleted using centrifugation at 5,000 g for 10 minutes. The supernatant was discarded and the tubes were drained on a paper towel. The pellet was resuspended in 3 ml of Cell Resuspension Solution, 3 ml of Cell Lysis Solution were added and gently mixed by inverting the tube. After three minutes of incubation at room temperature 5 ml of Neutralization Solution were added and mixed by inverting the tube. To ensure thorough lysate clearing the cell lysate was put in an upright position for 2-3 minutes. Lysates were poured into a PureYield™ Clearing Column placed in a new 50 ml tube and shortly incubated to collect cell debris on the top. All of the following centrifugation steps were performed at 1,500 g for indicated times. After centrifugation, the filtered lysate was poured on the PureYield™ Binding Column placed in a new 50 ml tube and centrifuged. The column bound plasmids were washed with 5 ml of Endotoxin Removal Wash solution and the flowthrough was discarded. Subsequently the column was washed with 20 ml of Column Wash Solution and after centrifugation the tip of the column was tapped on a paper towel to remove remaining ethanol. The elution of the plasmid was performed with 500 µl Nuclease-Free Water and transferred to a microcentrifugation tube. The concentration of the isolated plasmid was determined with a spectrophotometer (Nanodrop One C, Thermo Fisher Scientific, Waltham, MA, USA). The isolated plasmid was stored at -20 °C.

2.2. Two-Dimensional (2D) Cell Culture

All cell culture procedures were performed in laminar flow hoods under sterile working conditions. All cell lines were incubated in a humidified atmosphere at 37 °C, 5 % CO_2.

If not stated otherwise, the used medium was Dulbecco's Modified Eagle's Medium + GlutaMAX™-I (DMEM, Life Technologies, Carlsbad, CA, USA) which was

supplemented with 10 % foetal bovine serum (FBS, Life Technologies) and 1 % Penicillin-Streptomycin (Pen-Strep, Life Technologies).

2.2.1. Thawing of Cells

HEK293T (passage five, ATCC, Manassas, VA, USA) and L-Wnt3a (passage five, ATCC) cells were thawed 2-3 passages before use for further experiments. Nunc™ Cryo Tube™ Vials (Thermo Scientific) containing 3.1 × 10^6 HEK293T cells and 2 × 10^6 L-Wnt3a cells were taken out of liquid nitrogen and thawed in a 37 °C water bath and put into 5 ml pre-warmed DMEM (Life Technologies), supplemented with 10 % FBS (Life Technologies) and 1 % antibiotics (Pen-Strep, Life Technologies). The cells were centrifuged at 1,000 rpm for 5 minutes. After this step, the supernatant was discarded, and the pellet was resuspended in 10 ml of fresh culture medium. The cells were seeded into a 20 mm Tissue Culture Dish (CytoOne STARLAB International, Hamburg, Germany) and cultivated in the incubator at 37 °C in 5 % CO_2.

2.2.2. Passaging of Cells

In order to keep cells proliferating, splitting 1-3 times a week is an essential step. The splitting ratio for HEK293T was 1:5 and for L-Wnt3a 1:10. Therefore, the medium was removed from the dishes and the cells were rinsed with 1 × Dulbecco's Phosphate Buffered Saline (DPBS, Life Technologies). This step is important in order to remove FBS, which inhibits the activity of trypsin. After covering the cells with trypsin (LifeTech, Vienna, Austria) they were incubated for some minutes until all cells were detached. The cell detachment was monitored under the microscope. The detached cells were resuspended in DMEM (Life Technologies) containing 10 % FBS (Life Technologies) and 1 % antibiotics (Pen-Strep, Life Technologies), centrifuged for 5 minutes at 1,000 rpm and supernatants were discarded. The pellets were resuspended in fresh medium. According to the splitting ratio, a part of the cell suspension was transferred into a new tissue culture dish. For DNA isolation cells were washed twice with DPBS (Life Technologies) and the pellet was stored for DNA isolation (see section 2.3.1. DNA Isolation).

2.2.3. Freezing of Cells

Cryopreservation of cells was done in order to have a back-up of low passage number cells. In brief, cell suspension was prepared as described in paragraph 2.2.2., centrifuged for 5 minutes at 1,000 rpm and supernatant was discarded. According to cell count (performed on a NucleoCounter®NC-250™, Chemometec), the cell pellet was resuspended in an appropriate

amount of culture medium containing 10 % DMSO (Sigma-Aldrich) in Nunc™ Cryo Tube™ Vial (Thermo Scientific) in 1 ml aliquots transferred to a CoolCell® container (BioVision, Zurich, Switzerland) to ensure a standardised and controlled-rate of freezing of -1 °C/minute cell freezing in a -80 °C freezer. After storing the vials at -80 °C for one night, they were transferred into liquid nitrogen and kept there until further use.

2.2.4. Puromycin-Titration and Sulforhodamine B Colorimetric Assay

The titration of puromycin is a dose response experiment figuring out the minimum amount of the antibiotic required to kill all cells and after transfection with the BII-CMV-AfmW3A plasmid (Addgene), containing a puromycin resistance, to select for transfected cells. The puromycin titration was carried out for each cell line in duplicates. Therefore, 1×10^4 cells were seeded into 22 wells of a 96-well plate (Biologix, Pleasant Prairie, WI, USA) for each cell line. After one day of incubation, dilutions of puromycin (Sigma-Aldrich) were prepared and the following concentrations were added to the cells: 15 µg/ml, 10 µg/ml, 5 µg/ml, 4 µg/ml, 3 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml. In order to have a negative and positive control, each cell line contained one duplicate, which was treated with staurosporine (Fischer Scientific, Pittsburgh, PA, USA) and one duplicate untreated cells. Three days later, the Sulforhodamine B (SRB) colorimetric assay was carried out to determine the cytotoxicity of puromycin. SRB assay was performed as described before (Vichai and Kirtikara, 2006). In brief, the medium was removed from the cells, and they were washed two times with DPBS. After removing DPBS (Life Technologies) 100 µl of plain DMEM (Life Technologies) and 50 µl cold 10 % Trichloroacetic acid (Sigma-Aldrich) were added. After incubating the plates at 4 °C for one hour the plates were washed four times by submerging them in four different water baths. After each washing step the water was removed from the wells and the plates were air-dried at room temperature. On the next day 100 µl of 0.057 % SRB solution (Sigma-Aldrich) were added to each well and the plates were incubated for 30 minutes at room temperature. To remove unbound dye the plates were rinsed four times with 1 % acetic acid (Fisher Scientific). After removing the acetic acid, the plates were dried at room temperature. To solubilise protein-bound dye 200 µl of 10 mM unbuffered Tris base solution (Sigma-Aldrich) were added to each well and the plates were shaken for 5 minutes. Afterwards the fluorescence was measured at excitation wavelengths of 475 nm and at emission wavelengths of 580-640 nm in the GloMax® Explorer Multimode Microplate Reader (Promega, Madison, WI, USA).

2.3. Transfection

For the Transfection with the BII-CMV-AfmW3A plasmid three wells of a six-well culture plate (Biologix) were used for each cell-line (L-Wnt3a and HEK293T). To transfect the L-Wnt3a cells, 3.8×10^5 cells, for the HEK293T cells, 1.2×10^5 cells were seeded into each well of the 6-well plate (Biologix) in 2 ml of culture medium. At a cell confluence of 80 % each cell line was transfected in three conditions. One negative control, which was transfected with a Green Fluorescent Protein (GFP) plasmid (pmaxCloning™ Vector (Lonza, Basel, Switzerland)), acting as control for the selection with puromycin and two wells for the transfection with the BII-CMV-AfmW3A plasmid (Addgene) in two suggested ratios (1:2.5 and 1:5) with the Super PiggyBac Transposase expression vector (BioCat, Catalonia, Spain) according to the manufacturer's protocol. For the transfection with GFP, 0.5 µg of the GFP Plasmid were mixed with 8 µl of TurboFect (Life Technologies) and 50 µl of Opti-MEM (Fisher Scientific). The mixture was vortexed and incubated at room temperature for 15 minutes. To transfect the cells with the two plasmids BII-CMV-AfmW3A and the Super PiggyBac two mixtures were prepared. For the reaction mixture with the ratio 1:2.5, 0.2 µg of Super PiggyBac Transposase expression vector (BioCat) were combined with 0.5 µg of the BII-CMV-AfmW3A plasmid (Addgene), 8 µl of TurboFect (Life Technologies) and 50 µl of Opti-MEM (Fisher Scientific). For the reaction mixture with the ratio 1:5, 0.2 µg of Super piggyBac Transposase expression vector were combined with 1 µg of the BII-CMV-AfmW3A plasmid (Addgene), 8 µl of TurboFect (Life Technologies) and 50 µl of Opti-MEM (Fisher Scientific). The mixtures were vortexed and incubated for 15 minutes at room temperature. After the incubation time, the prepared transfection reagent was carefully added to the culture well and mixed gently. Approximately 24-48 hours later the medium of the cell culture was removed and replaced with fresh medium containing Puromycin (Sigma-Aldrich) to start the selection. The transfected HEK293T cells and L-Wnt3a cells were selected with 1 µg/ml and 10 µg/ml, respectively.

2.3.1. DNA Isolation

DNA extraction was performed using the ReliaPrep™ gDNA Tissue kit (Promega, Madison, WI, USA) according to the instructions of the manufacturer. In brief, the frozen pellet (see paragraph 2.2.2.) was mixed with 160 µl of DPBS (Life Technologies) and 20 µl of Proteinase K Solution and 200 µl of Cell Lysis Buffer were added and mixed by vortexing for 10 seconds. After incubating the sample at 56 °C for 30 minutes at 600 rpm, 200 µl of RNase A Solution were added and again mixed by vortexing for 10 seconds. The sample was further

incubated at 56 °C for 10 minutes at 600 rpm and 250 µl of Binding Buffer were added and mixed for 10 seconds. The sample was transferred onto the membrane of the ReliaPrep™ Binding Column placed in a collection tube and centrifuged for 1 minute at maximum speed. The flowthrough was discarded, and the column was placed into a new collection tube and 500 µl of Column Wash Solution were added on the membrane before centrifuging the sample for 2 minutes at maximum speed. This step was repeated twice. Afterwards, the column was transferred into a microcentrifuge tube and the DNA was eluted using 50 µl Nuclease-Free Water by centrifugation for 1 minute at maximum speed. The concentration of the DNA was measured on a spectrophotometer (Nanodrop One C, Thermo Fisher Scientific) and samples were stored at -20 °C.

2.3.2. PCR

The Polymerase chain reaction (PCR) was performed in a volume of 20 µl. The PCR master mix was prepared with LongAmp® *Taq* 2 × Master Mix (New England Biolabs, Ipswich, MA, USA), primers (10 µM), Betaine (5 M) (Sigma-Aldrich), DMSO (3 % final) (Sigma-Aldrich) and PCR-grade water according to Table 1. The primers were purchased from Sigma-Aldrich (Merck):

Afm_Wnt3a_fw: ACTGGTGACGAAAGTCAAGC
Afm_Wnt3a_rv: AAGTATCCGAGTGGGGCCAT

Table 1: *Master Mix Composition*

	for 1 reaction
LongAmp® Tag 2 × Master Mix	10 µl
Primer forward (fw) (10 µM)	0.8 µl
Primer reverse (rv) (10 µM)	0.8 µl
Betaine (5 M)	2 µl
DMSO – 3 % final	0.6 µl
Nuclease-Free Water	4.8 µl
DNA	1 µl
Total	20 µl

The amplification was carried out in a Mastercycler pro S (Eppendorf, Hamburg, Germany), which uses the vapo.protect™ technology according to the cycling scheme in Table 2. The PCR products and the GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific) were mixed with 6 × DNA Loading Dye (Thermo Scientific) and analysed on a 1.5 % agarose gel stained with Atlas ClearSight DNA Stain (Bioatlas, Tartu, Estonia). The agarose gel was run for approximately one hour. The results were evaluated with the ChemiDoc™ XRS (Bio-Rad, Hercules, CA, USA).

Table 2: *PCR Cycling Scheme*

Temperature	Time	Number of cycles
94 °C	3 min	1 cycle
60 °C	30 sec	1 cycle
65 °C	3 min	1 cycle
94 °C	30 sec	40 cycles
54 °C	30 sec	
65 °C	1 min	
65 °C	10 min	1 cycle
4 °C	∞	1 cycle

2.3.3. Gel Extraction and Sequencing

To extract the DNA from the agarose gel, to have it sequenced, the GeneJET Gel Extraction Kit (Thermo Fisher Scientific) was carried out according to the manufacturer's instructions. In brief, the DNA fragment was excised by cutting a gel slice containing the fragment with a blade. This slice was transferred into a microcentrifuge tube and its weight was recorded. Binding Buffer was added 1:1 (volume : weight) to the gel slice and the mix was incubated at 55 °C for 10 minutes. After mixing the tube by vortexing, 800 µl of the gel solution was pipetted on the membrane of the GeneJET purification column and centrifuged for 1 minute at 13,000 g. The flowthrough was discarded, and the column was placed back in the same collection tube. 100 µl of the Binding Buffer were transferred on the GeneJET purification column and centrifuged for 1 minute at 13,000 g. Again, the flowthrough was discarded, and the column

was placed back into the same collection tube before adding 700 µl of Wash Buffer to the membrane and centrifuging the sample for 1 minute at 13,000 g. The flowthrough was discarded, and the column was placed back into the same tube. Another centrifugation step for 1 minute at 13,000 g is necessary to avoid alcohol in the purified DNA solution because ethanol may inhibit the sequencing reactions. Later on, the Gene JET purification column was transferred into a clean microcentrifuge tube and DNA was eluted with 30 µl of Nuclease-Free Water by centrifugation for 1 minute at 13,000 g. The DNA concentration was determined on a spectrophotometer (Nanodrop One C, Thermo Fisher Scientific) and sent to Microsynth AG (Balgach, Switzerland) for sequencing at a concentration of 18 ng/100 bp of amplicon length.

2.4. Collection of Conditioned Media

For the Collection of the conditioned media, following conditions existed: L-Wnt3a not treated (nt), L-Wnt3a transfected with a 1:2.5 and 1:5 dilution of the BII-CMV-AfmW3A Plasmid (Addgene), HEK293T not treated (nt), HEK293T transfected with a 1:2.5 and 1:5 dilution of the BII-CMV-AfmW3A Plasmid (Addgene). To collect the conditioned media 3.75×10^6 HEK293T and L-Wnt3a cells were seeded into a T75 cell culture flask (Thermo Scientific) for each condition. Therefore 10 ml DMEM (Life Technologies) containing 10 % FBS (Life Technologies) and 1 % antibiotics (Pen-Strep, Life Technologies) were used. After culturing for one day the cells were washed with 10 ml DPBS (Life Technologies) two times and the medium was changed to 22.5 ml basal medium, consisting of Advanced DMEM/F-12 (Fisher Scientific), GlutaMAX™ (Fisher Scientific), HEPES Buffer Solution (Life Technologies) and antibiotics (Pen-Strep, Life Technologies) (Table 3). After seven days of incubation the medium was transferred into a 50 ml tube and centrifuged at 8 °C for 5 minutes at 400 g. Afterwards the supernatant was sterile filtrated through a syringe filter with a pore size of 0.22 µm (neoLab, Seoul, South Korea). At the end, six different conditioned media were produced and stored at 4 °C until further use.

***Table 3**: Basal Medium Composition*

Components	Stock	Amount	Final concentration
Advanced DMEM/F-12		500 ml	
GlutaMAX™	100 ×	5 ml	1 ×
HEPES	1 M	5 ml	10 mM
Antibiotics (Pen-Strep)	100 ×	5 ml	1 ×

In order to cultivate organoids with them, the conditioned media has to be supplemented to the refined medium (Table 4). Therefore, the refined medium was prepared without Wnt3a conditioned media and divided into six 15 ml tubes, each containing 4 ml of the medium. Afterwards 4 ml of each conditioned medium were added and stored at 4 °C.

***Table 4**: Refined Medium Composition*

Components	Stock	Amount	Final concentration
Basal Medium		18.475 ml	
B27 (Life Technologies)	50 ×	1 ml	1 ×
n-Acetylcysteine (Sigma-Aldrich)	500 mM	100 µl	1 mM
A83-01 (Bio-Techne, MN, USA)	50 µM	50 µl	500 nM
Gastrin (Sigma-Aldrich)	10 µM	50 µl	10 nM
HGF (PeproTech, NJ, USA)	50 µg/ml	50 µl	50 ng/ml
hIGF1 (PeproTech)	100 µg/ml	50 µl	100 ng/ml
hFGF2 (PeproTech)	100 µg/ml	25 µl	50 ng/ml
mNoggin (PeproTech)	50 µg/ml	100 µl	100 ng/ml
R-spondin c.m.	100 %	5 ml	10 %
Gentamycin (Sigma-Aldrich)	10 mg/ml	100 µl	20 µg/ml
Divide into 6 × 4 ml aliquots			
Wnt3a c.m. (each preparation)	100 %	4 ml	50 %

2.5. Three-Dimensional (3D) Cell Culture: Organoids

All cell culture procedures were handled in a laminar flow under sterile working conditions. All incubation steps were carried out in a humidified atmosphere at 37 °C and 5 % CO_2.

2.5.1. Isolation of Adult Intestinal Stem Cells

For the isolation of stem cells 10-20 biopsies were taken out of coon tissue using endoscopic biopsy forceps and transferred into 15 ml tubes filled with Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich), 200 µg/ml Gentamycin (Sigma-Aldrich) and 1 % Pen-Strep (Sigma-Aldrich). Afterwards the samples were washed with HBSS (Sigma-Aldrich), Gentamycin (Sigma-Aldrich) and Pen-Strep (Sigma-Aldrich) until they were cleared off debris. To isolate the crypts the tissue was incubated in 10 mM EDTA (Sigma-Aldrich) in DPBS (Life Technologies) for 60 minutes and resuspended every 10 minutes using 5 ml pipettes. After the crypt yield was good, supernatant was transferred in 50 ml tubes which were precoated with DPBS (Life Technologies) containing 10 % FBS (Life Technologies). The crypts were centrifuged for 5 minutes at 400 g and supernatant containing tissue debris was removed. The samples were resuspended in 30 ml DPBS (Life Technologies), and 1 ml of the mixture was transferred into a microcentrifuge tube for cell seeding. The tube was centrifuged for 5 minutes at 400 g and the supernatant was removed. The pellet was resuspended in basal medium (20 µl/well), mixed with 30 µl/well of extracellular matrix (Geltrex™, Fisher Scientific) and 50 µl of the mixture were seeded in approximately six little drops in one well of a 24-well plate (Biologix). After 15 minutes of polymerisation at 37 °C the drops were overlayed with 750 µl of refined medium which was changed every second to third day.

2.5.2. Passaging Intestinal Organoids

Organoids are passaged when they demonstrate a dark core of sloughed off enterocytes or when they are too big using a split ratio between 1:2 and 1:5. Therefore, the medium was used to detach the drops of Geltrex™ (Fisher Scientific) of 24-well plate and transferred into a 15 ml tube. After all the organoids were settled on the bottom, approximately half of the supernatant was removed, and fresh basal medium was added to the tube. Using a glass Pasteur pipette fire-narrowed from 1.5 mm to about 0.5 mm the organoids were pipetted up and down until their breaking could be macroscopically seen. Afterwards the organoids were centrifuged for 5 minutes at 80 g and 8 °C, supernatant was discarded carefully, and the pellet was kept on ice. For a single well the pellet was resuspended in 20 µl of basal medium and 30 µl of cooled

Geltrex™ (Fisher Scientific). After taking a pre-warmed 24-well plate (Biologix) out of the incubator 50 µl of the organoid suspension were placed in little drops in each well. It takes about 15 minutes at 37 °C to solidify the basement matrix. It is important to put the 24-well plate upside down into the incubator during this incubation step. Subsequently the drops were overlayed with 750 µl of pre-warmed refined medium and the plate was transferred to the incubator. The medium was changed three times a week every 2-3 days.

2.6. Testing of Different Conditioned Media

2.6.1. Wnt Reporter Activation

To test the Wnt activation, three wells with 1 × 10^6 HEK293T were seeded in a 6-well plate (Biologix). After one day of cultivation the cells were transfected. Therefore, in a microcentrifuge tube 260 µl of Opti-MEM (Fisher Scientific) were mixed with 1.8 µg of pSTF (Addgene) and 0.112 µg of pIS1 (Addgene) before the addition of 3.6 µl of TurboFect (Life Technologies). After incubation for 15 minutes at room temperature, 260 µl of the mixture was added to each well of the 6-well plate (Biologix) drop by drop. One well was left untouched in order to serve as a negative control. The plate was put in the incubator for 24 hours.

After the incubation time, the cells were harvested, and the transfected cells were re-seeded in twelve wells of a 48-well plate (Biologix). For the negative control only one quarter of the cells were re-seeded in three wells of a 48-well plate. The cells were left in the incubator overnight. On the next day 400 µl of basal medium were mixed with 400 µl of the conditioned media. After removing the medium for each condition, the wells were treated with 250 µl of the prepared conditioned media (triplicates). Three wells with HEK293T and basal medium served as negative control. The cells were incubated overnight.

The next day 100 µl of the 1 × Passive Lysis Buffer (Promega) were added to each well after removing the culture medium and the culture plates were placed on a rocking platform and incubated at room temperature for 30 minutes. Afterwards the lysates were transferred into microcentrifuge tubes and snap-frozen in liquid nitrogen and stored at -20 °C. The measurement was performed in duplicates according to the instructions of the Dual-Luciferase® Reporter Assay System (Promega). In brief, the Luciferase Assay Reagent II (LAR II) was prepared by resuspending the lyophilized Luciferase Assay Substrate in 10 ml of Luciferase Assay Buffer II and then the reagent was aliquoted and stored at -80 °C. To prepare

the Stop & Glo® Reagent one volume of 50 × Stop & Glo® Substrate was mixed with 50 volumes of Stop & Glo® Buffer in a glass tube. Before measuring the plate with the GloMax plate reader (Promega) the reagents were equilibrated to room temperature whilst 30 µl of cell lysate was prepared in duplicates into a white 96-well plate (Greiner Bio-One, Austria). The plate reader was pre-programmed to perform a 2-second premeasurement delay, followed by a 10-second measurement period for each assay. Reagent injectors were used to dispense 25 µl of LAR II and 25 µl of Stop & Glo® Reagent into each well. Priming the injectors before measuring helps to prevent dilution and contamination of the primed reagents. For the evaluation of the results Microsoft Excel, Version 2103, and GraphPad Prism, Version 5, were used.

2.6.2. Viability Assay

Viability of organoids was assessed using the RealTime-Glo™ MT Cell Viability Assay (Promega). Organoids were trypsinised in order to get single cells. The cells were counted with the NucleoCounter®NC-250™ (ChemoMetec, Denmark) and 150,000 cells were seeded in each of 4 wells of a 24-well plate (Biologix) with refined medium. After three days of cultivation, the organoids were harvested, counted and 50 organoids per well were re-seeded in six wells of a white 96-well plate (Greiner Bio-One) for each condition (HEK293T not treated (nt), HEK293T 1:2.5, HEK293T 1:5, L-Wnt3a nt, L-Wnt3a 1:2.5, L-Wnt3a 1:5). For a background control every condition had three wells without organoids but with 100 µl of refined medium supplemented with the different conditioned media produced by HEK293T and L-Wnt3a cells. The detection reagents were prepared according to the instructions of the manufacturer and added to all wells. In brief, for one well 99.8 µl of conditioned media were mixed with 0.1 µl of NanoLuc® Enzyme and 0.1 µl of MT Cell Viability Substrate. Later the organoids were overlayed with 100 µl of the prepared media per well. The luminescence was measured 0 h, 24 h, 48 h, 72 h and 96 h after adding the substrate using a GloMax plate reader (Promega). To evaluate the results Microsoft Excel, Version 2103, and GraphPad Prism, Version 5, were used.

2.6.3. Apoptosis Assay

Apoptosis and necrosis were assessed by RealTime-Glo Annexin V Apoptosis Assay (Promega). As described before, 50 organoids per well were re-seeded in six wells of a white 96-well plate (Greiner Bio-One) for each condition (HEK293T nt, HEK293T 1:2.5, HEK293T

1:5, L-Wnt3a nt, L-Wnt3a 1:2.5, L-Wnt3a 1:5). For a background control every condition had three wells without organoids but with 100 µl of conditioned medium. The detection reagents were prepared according to the instructions of the manufacturer and added to all wells. In brief, for one well 99.5 µl conditioned media were mixed with 0.1 µl of Annexin V-LgBiT, 0.1 µl of Annexin V-SmBiT, 0.1 µl of $CaCl_2$, 0,1 µl of Annexin V NanoBiT® Substrate and 0.1 µl of Necrosis Detection Reagent. Later the organoids were overlayed with 100 µl of the prepared media per well. 0 h, 24 h, 48 h, 72 h and 96 h after adding the substrate the luminescence (apoptosis) and the fluorescence (necrosis) was measured using a GloMax plate reader (Promega). The fluorescence was measured at excitation wavelengths of 475 nm and at emission wavelengths of 500-550 nm. To evaluate the results Microsoft Excel, Version 2103, and GraphPad Prism, Version 5, were used.

2.6.4. Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

In order to concentrate the conditioned media, 1 ml of each conditioned medium was transferred in a microcentrifuge tube and put in a Concentrator 5301 (Eppendorf) for a ten-times concentration (100 µl sample volume).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 10 % gels with 1.5 mm thickness. For the separating gel buffer 1.5 M Tris (Sigma-Aldrich) (pH 8.8) and 0.4 % SDS (w/v) (Sigma-Aldrich) and for the stacking gel buffer 0.5 M Tris (Sigma-Aldrich) (pH 6.8) and 0.4 % SDS (w/v) were used.

For the 10 % gels following mixture was used (Table 5):

Table 5: *SDS-PAGE Gel Composition*

For 2 gels	10 % Separating Gel	Stacking gel
Aqua dest.	6.71 ml	3.4 ml
30 % Acrylamide/Bis-acrylamide Solution (Carl Roth, KA, Germany)	6.03 ml	0.83 ml
Gel buffer	4.68 ml	0.68 ml
10 % APS Solution (BioRad)	150 µl	50 µl
TEMED (Sigma-Aldrich)	15 µl	5 µl

APS-Solution (BioRad) and TEMED (Sigma-Aldrich) were added just before casting. After preparing the gel casting stand with appropriate short and spacer plates (BioRad), the stand was filled with separating gel up to about 5 cm and overlayed with Isopropanol (Sigma-Aldrich) in order to prevent contact with oxygen which inhibits the polymerisation and to make the border between separating and stacking gel sharper. After 30 minutes the gel was polymerised, and isopropyl alcohol was rinsed out with Aqua dest. and the excess water was tapped off with filter paper. Afterwards APS (BioRad) and TEMED (Sigma-Aldrich) were added to the stacking gel and poured on top of the polymerized separating gel and the correct comb (1.5 mm) was inserted. After 20 minutes of polymerisation, gels were assembled in the electrode assembly and clamping frame. The inner and outer chamber were filled with 1 × SDS running buffer (prepared from 10 × SDS-PAGE running buffer, Table 6).

***Table 6**: 10 × SDS-PAGE Running Buffer*

25 mM Tris (Sigma-Aldrich)	15.02 g
200 mM Glycine (Sigma-Aldrich)	78,1 g
0.1 % (w/v) SDS (Sigma-Aldrich)	5 g
Aqua dest.	Ad 500 ml

During the polymerisation steps the samples were prepared. Therefore 135 µl of 3 × SDS Blue loading buffer (New England Biolabs) were mixed with 15 µl of 30 × Reducing Agent DTT (New England Biolabs). The prepared loading buffer (10 µl) was added to 20 µl of the samples and incubated for 5 minutes at 99 °C, cooled down and spun down before loading on the gel with special gel loading tips in order to avoid a carryover of samples in neighbouring slots. In the first and last slot of every gel Precision Plus Protein Dual Color Standards (BioRad) were loaded. Protein separation was performed at 100 V until all samples were through the stacking gel. Subsequently the setting was changed to 150 V and after 1.5-2 hours the run was stopped.

For protein staining on polyacrylamide gels two procedures were used. The first one was an ultrasensitive silver stain system which was performed according to the instructions of the Pierce Silver Stain Kit (Thermo Fisher Scientific). In brief, the gel was washed two times for 5 minutes in ultrapure water. Afterwards the gel was fixed by placing the gel in 30 % ethanol : 10 % acetic acid solution for 15 minutes. This step was repeated once

and subsequently the gel was washed two times in 10 % ethanol and two times for five minutes in ultrapure water. In the meantime, the Sensitizer Working Solution was prepared by mixing 50 µl of Sensitizer with 25 ml of water and after the washing steps the gel was incubated in this solution for one minute. The gel was washed two times for one minute with water and the Stain Working Solution was prepared by mixing 500 µl of Enhancer with 25 ml of Stain. The gel was stained for 30 minutes. During this incubation time the Developer Working Solution was prepared by adding 500 µl of Enhancer to 25 ml of developer. After washing the gel two times for 20 seconds with ultrapure water, the developer was added to the gel. As soon as bands appeared the reaction was stopped by overlaying the gel with 5 % acetic acid for ten minutes.

The second gel was stained with the PageBlue™ Protein Staining Solution (Thermo Scientific) according to the manufacturer's instructions. In brief, the gel was washed three times with 150 ml of ultrapure water for 10 minutes. Gels were relocated into 20 ml of the PageBlue™ Protein Staining Solution and incubated for 60 minutes at room temperature on a shaking platform. After this incubation time, the gel was destained in ultrapure water until clear bands were visible.

Gel documentation was performed on the ChemiDoc™ XRS (BioRad).

3. Results

3.1. Transfection

To determine if the transfection has worked, the GFP control was evaluated with fluorescence microscopy one day after the transfection. This is shown in Figure 4. About 10 % of the L-Wnt3a cells were transfected with GFP, whereas approximately 50 % of the HEK293T cells took up the GFP plasmid.

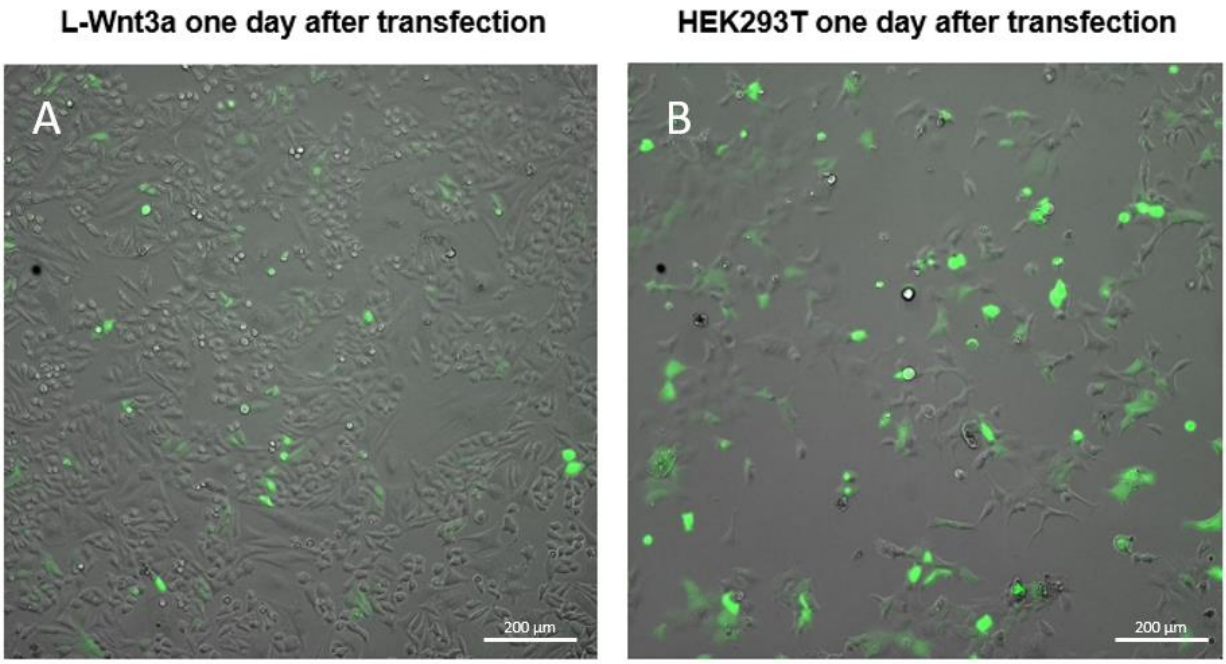

Figure 4: *Assessment of lipid-mediated transfection efficiencies using GFP visualization. The cell lines L-Wnt3a (A) and HEK293T (B) were transfected with the GFP plasmid. 24 hours after the transfection pictures were taken.*

3.2. PCR

After isolating DNA from the transfected and not-transfected cells a PCR was performed to check if the integration of the plasmid was successful. As can be seen in the picture of the agarose gel after electrophoresis (Figure 5) there are PCR bands with a length of 131 bp showing that in all transfected cells the plasmid was integrated. This happened independently of the different ratios between the Super PiggyBac Transposase expression vector and the BII-CMV-AfmW3A plasmid. Moreover, the positive control (PCR of BII-CMV-AfmW3A plasmid DNA) shows a band similarly to the ones of the transfected cells, whereas the negative control (Nuclease-Free Water) is negative. Also, the results of the sequencing showed that using these primers 100 % homology between the sequences of the amplified DNA fragments and the plasmid sequence was achieved.

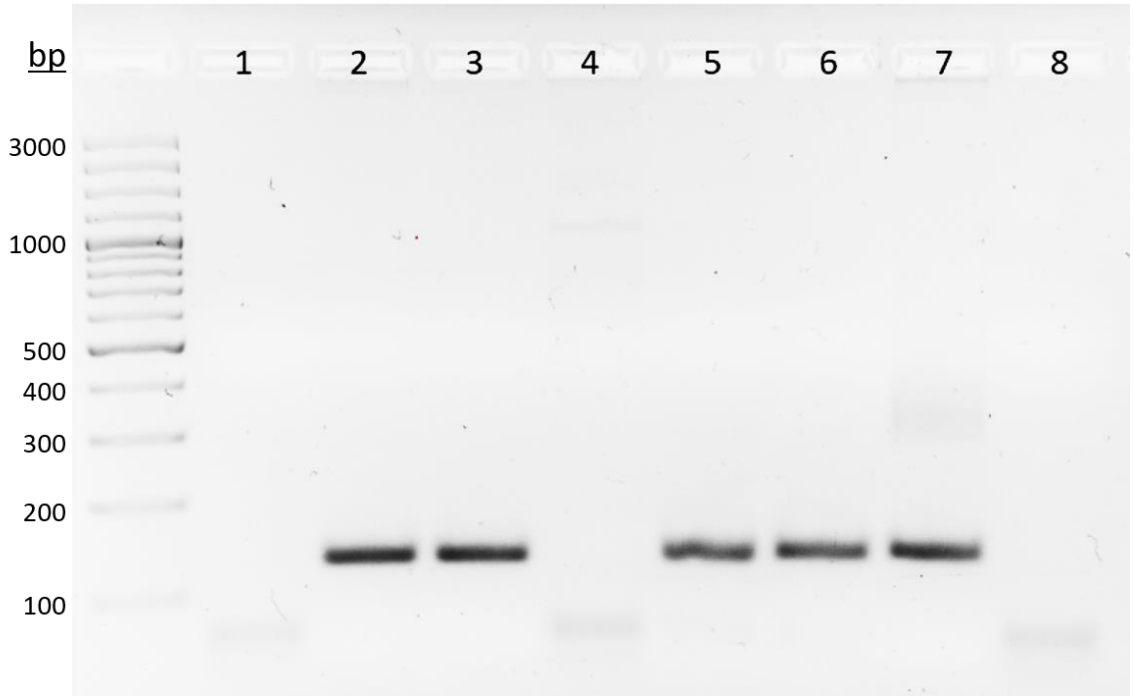

Figure 5: *Agarose gel electrophoresis of PCR products. Lane 1: HEK293T nt; Lane 2: HEK293T 1:2.5; Lane 3: HEK293T 1:5; Lane 4: L-Wnt3a nt; Lane 5: L-Wnt3a 1:2.5; Lane 6: L-Wnt3a 1:5; Lane 7: positive control; Lane 8: negative control.*

3.3. Wnt Reporter Activation

Next, we tested which of the conditioned media produced by HEK293T and L-Wnt3a cells (HEK nt, HEK293T 1:2.5, HEK293T 1:5, L-Wnt3a nt, L-Wnt3a 1:2.5, L-Wnt3a 1:5) are able to activate canonical Wnt signalling in HEK293T cells. Therefore, HEK293T cells were transfected with a reporter vector and incubated with the different conditioned media. Some cells were cultivated in medium obtained from L-Wnt3a cells cultivated in FBS as a reference, whereas the negative control was overlayed with basal medium. The measurement was performed in duplicates according to the instructions of the Dual-Luciferase® Reporter Assay System. As can be seen in Figure 6 the media produced by the HEK293T and L-Wnt3a showed less activation of the canonical Wnt signalling as the conditioned media obtained from the L-Wnt3a cells cultivated in FBS, which serves as a positive control. Interestingly, all conditioned media, except L-Wnt3a nt, showed about the same ratio between Firefly and Renilla luciferases. Even though L-Wnt3a cells were cultured without FBS, L-Wnt3a nt was able to activate the reporter gene more than the other conditioned media.

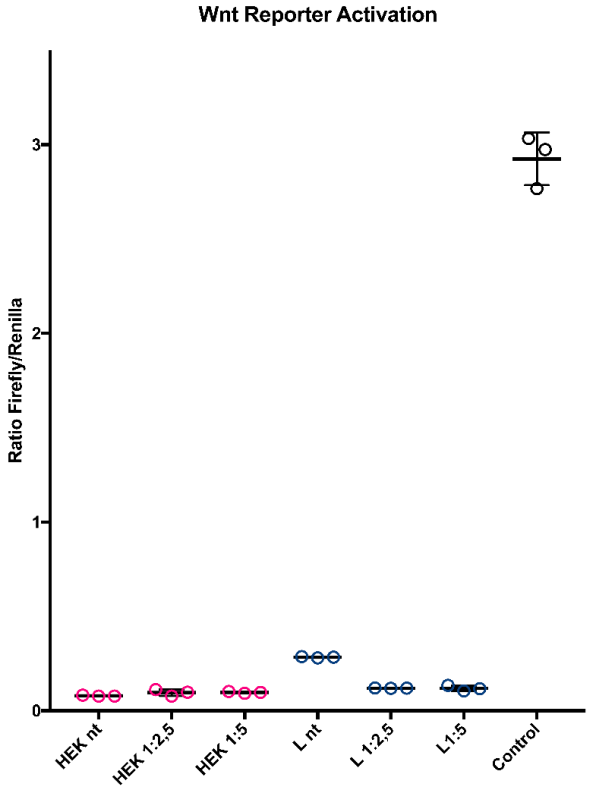

Figure 6: *Dual-Luciferase® Reporter Assay. The graph shows the ratios between firerfly luciferase und renilla luciferase. The result shows that only HEK293T cells treated with the control activate the canonical Wnt pathway. Cells cultivated with the different c.m. produced by HEK293T and L-Wnt3a cannot activate this cascade.*

3.4. Cell Viability and Cell Death

Because of the results of the Wnt reporter activation assay, we looked at the viability, apoptosis and necrosis of canine colon organoids cultivated with the different conditioned media. After preparing the detection reagents and mixing them with the conditioned media as described above, the organoids were overlayed with the media. 0 h, 24 h, 48 h, 72 h and 96 h the luminescence was measured. Three wells without organoids, but with medium, serve as a background control for every medium condition. The data was normalised to the measured luminescence/fluorescence of the conditioned media produced by L-Wnt3a cells cultivated in medium containing FBS (positive control) for easier interpretation of the data. As can be seen in Figure 7 the organoids treated with the positive control were the most viable closely followed by the organoids treated with the L-Wnt3a nt conditioned medium. Interestingly, the organoids cultivated in medium produced by the HEK293T nt were the ones showing the lowest viability. To sum up, organoids treated with the conditioned media produced by the L-Wnt3a cells were more viable than the organoids treated with the conditioned media produced by HEK293T. But none of the conditioned media reached such a high viability as the positive control. Regarding the data obtained from the RealTime-Glo Annexin V Apoptosis Assay it should be noted that most of the time organoids treated with the positive control medium were the most apoptotic. The data for necrosis reveal that the organoids treated with the conditioned media were more necrotic than the ones cultured in the positive control medium, suggesting that the organoids treated with the positive control undergo more apoptosis than necrosis whereas with organoids cultured in conditioned media it is exactly the other way round. Canine colon organoids cultured in conditioned medium produced by HEK293T 1:2.5 had the lowest rate of apoptotic cells. The treatment with L-Wnt3a 1:2.5 led to the lowest necrosis and to the second lowest apoptosis rate.

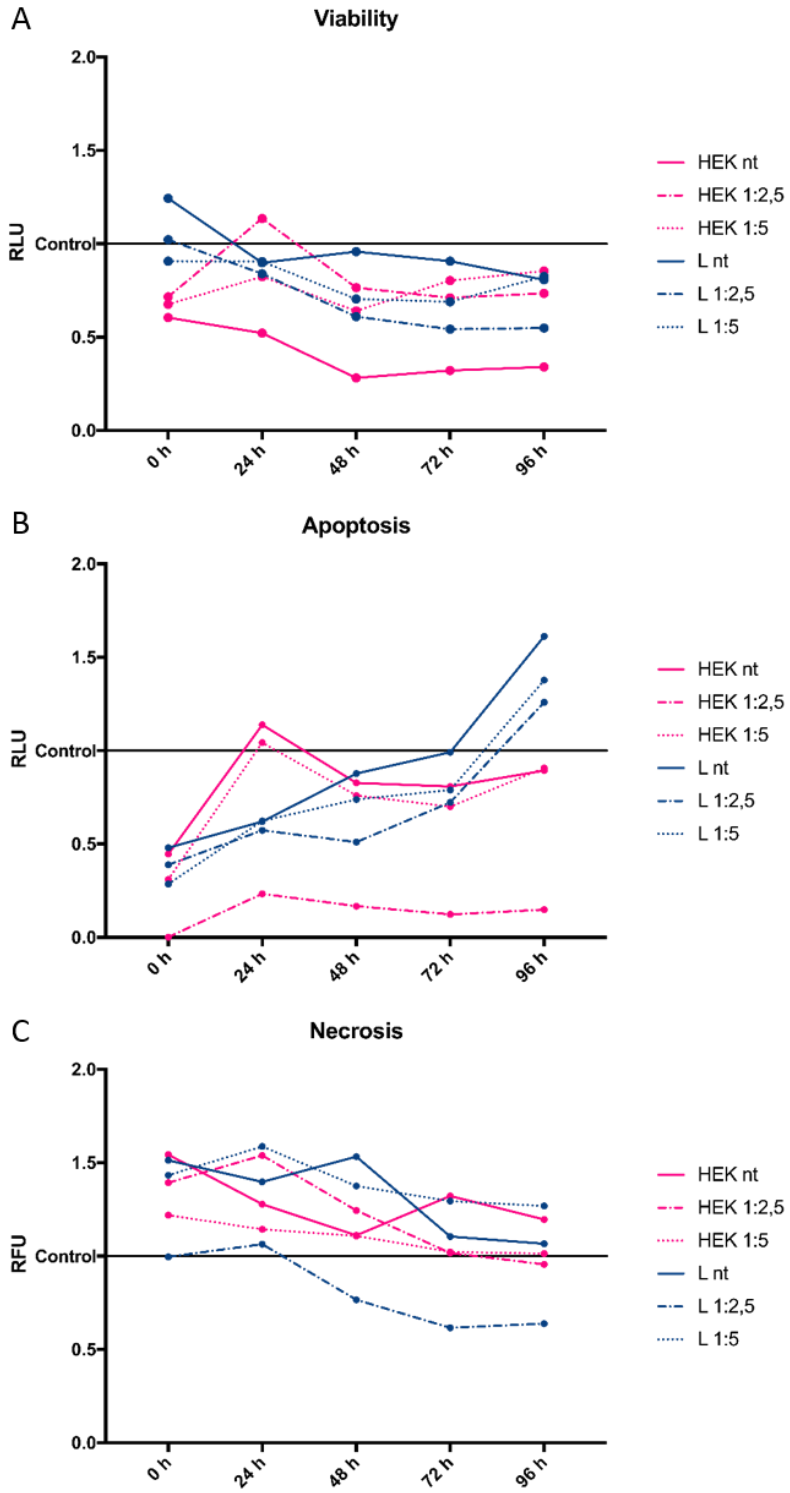

Figure 7: *Relative luciferase activity of cell viability assay (A) and cell apoptosis assay (B) after 0, 24, 48, 72, 96 hours after the supplementation of substrate to colon organoids in the different c.m. produced by HEK293T (pink) and L-Wnt3a (blue) cells. (C) Relative fluorescence activity of cell necrosis assay after 0, 24, 48, 72, 96 hours after the supplementation of substrate to colon organoids in the different c.m. produced by HEK293T (pink) and L-Wnt3a (blue) cells.*

3.5. SDS-PAGE

Next an SDS-PAGE was performed to determine if the Afamin-Wnt3a complex is present in the conditioned media. Therefore, we first concentrated the conditioned media, but the concentration was too high, and it was not possible to get interpretable bands. Hence, the non-concentrated samples were used. As can be seen on both gels, each conditioned medium contained proteins with a size between 50 kDa and 75 kDa. Conditioned medium obtained from L-Wnt3a cells cultivated in medium containing FBS was used as a positive control. However, also the positive control showed one band between 50 kDa and 75 kDa but the silver staining showed a wider band at the positive control than at the other conditioned media. The line of the positive control also reveals a weak band at the height of 37 kDa.

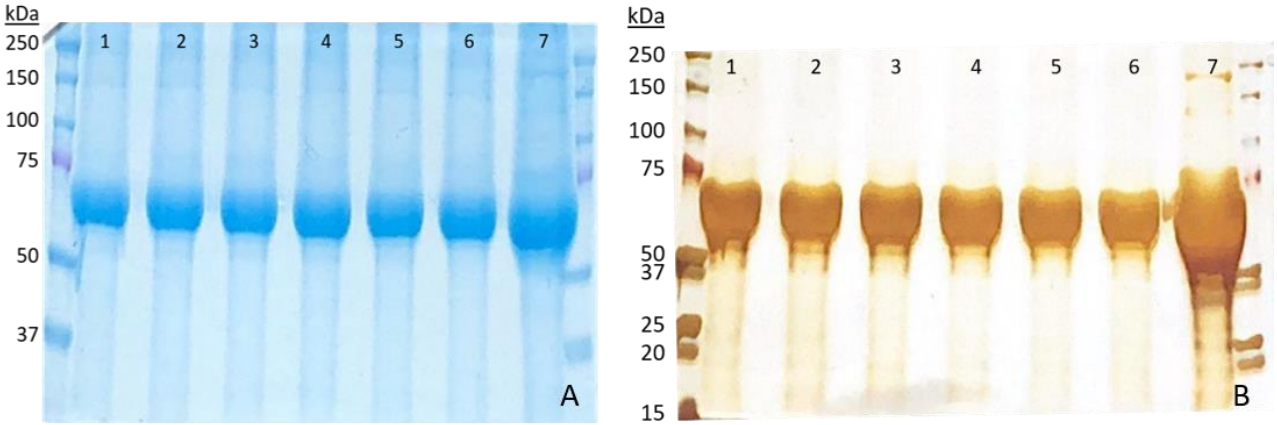

Figure 8: *SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins from different c.m. Lane 1: HEK293T nt; Lane 2: HEK293T 1:2.5; Lane 3: HEK293T 1:5; Lane 4: L-Wnt3a nt; Lane 5: L-Wnt3a 1:2.5; Lane 6: L-Wnt3a 1:5; Lane 7: positive control. Gel A was stained with PageBlue™ Protein Staining Solution. Gel B was stained with Pierce Silver Stain Kit.*

3.6. Organoid Culture

To verify the results of the viability assay canine colon organoids were cultivated in the different conditioned media for 28 days. In order to be able to compare the conditioned media, some organoids were cultivated in refined medium derived from FBS-containing Wnt3a conditioned medium as a positive control. Conditioned media produced by not-transfected cells (HEK293T nt and L-Wnt3a nt) were used as the negative control. Figure 9 shows that the organoids cultivated in refined medium had a normal growth rate and most of the time budding structures. It can also be shown in Figure 9, 10 and 11. that all organoids survived the 28-day period of cultivation. Interestingly, organoids treated with the different conditioned media had a slower growth rate than the one treated with the refined medium as can be seen by the difference in passage at the end of day 28. While control organoids were passaged four times (i.e. p.15), organoids treated with conditioned media were split only twice. The majority of the organoids cultivated in the different conditioned media were lacking budding structures.

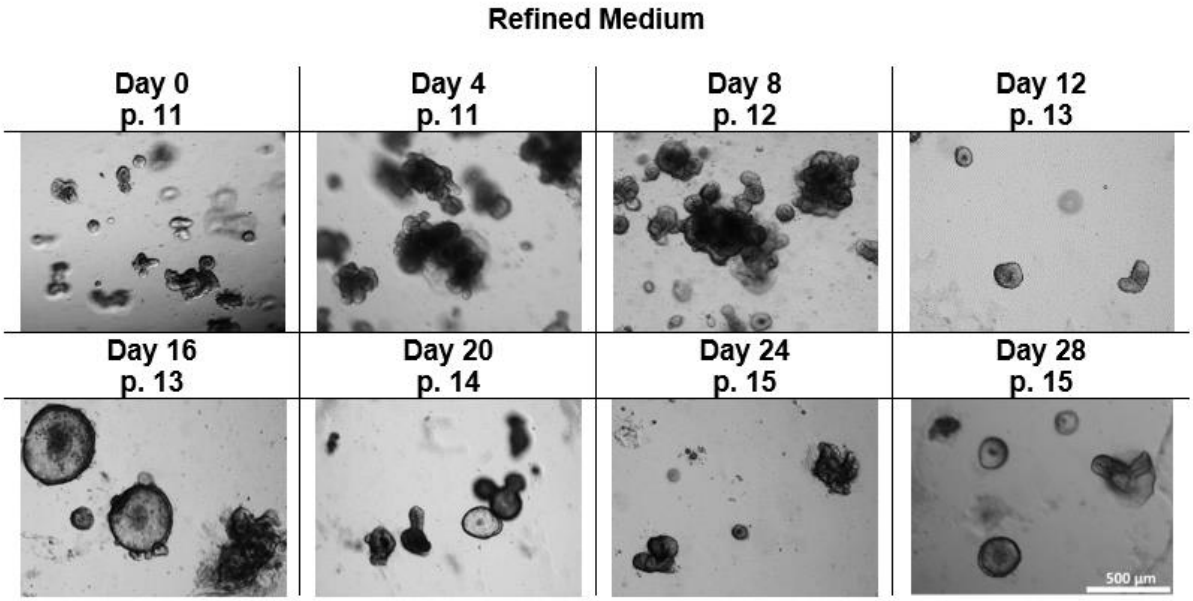

Figure 9: *Light microscopic images of colon organoids cultivated in refined medium for 28 days; scale bar represents 500 µm. p = passage*

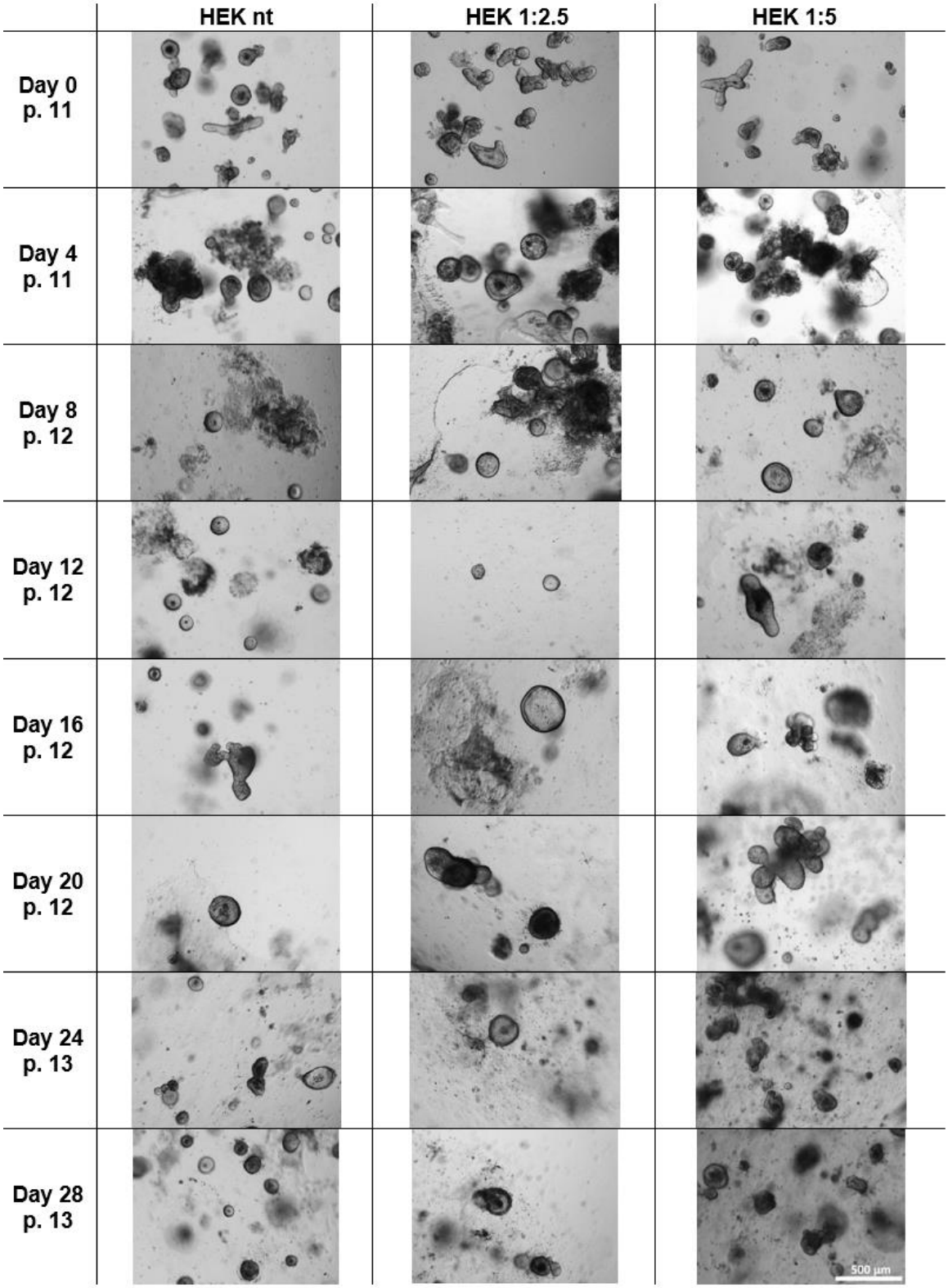

Figure 10: *Light microscopic images of colon organoids cultivated in c.m. produced by HEK293T cells for 28 days; scale bar represents 500 µm. p = passage*

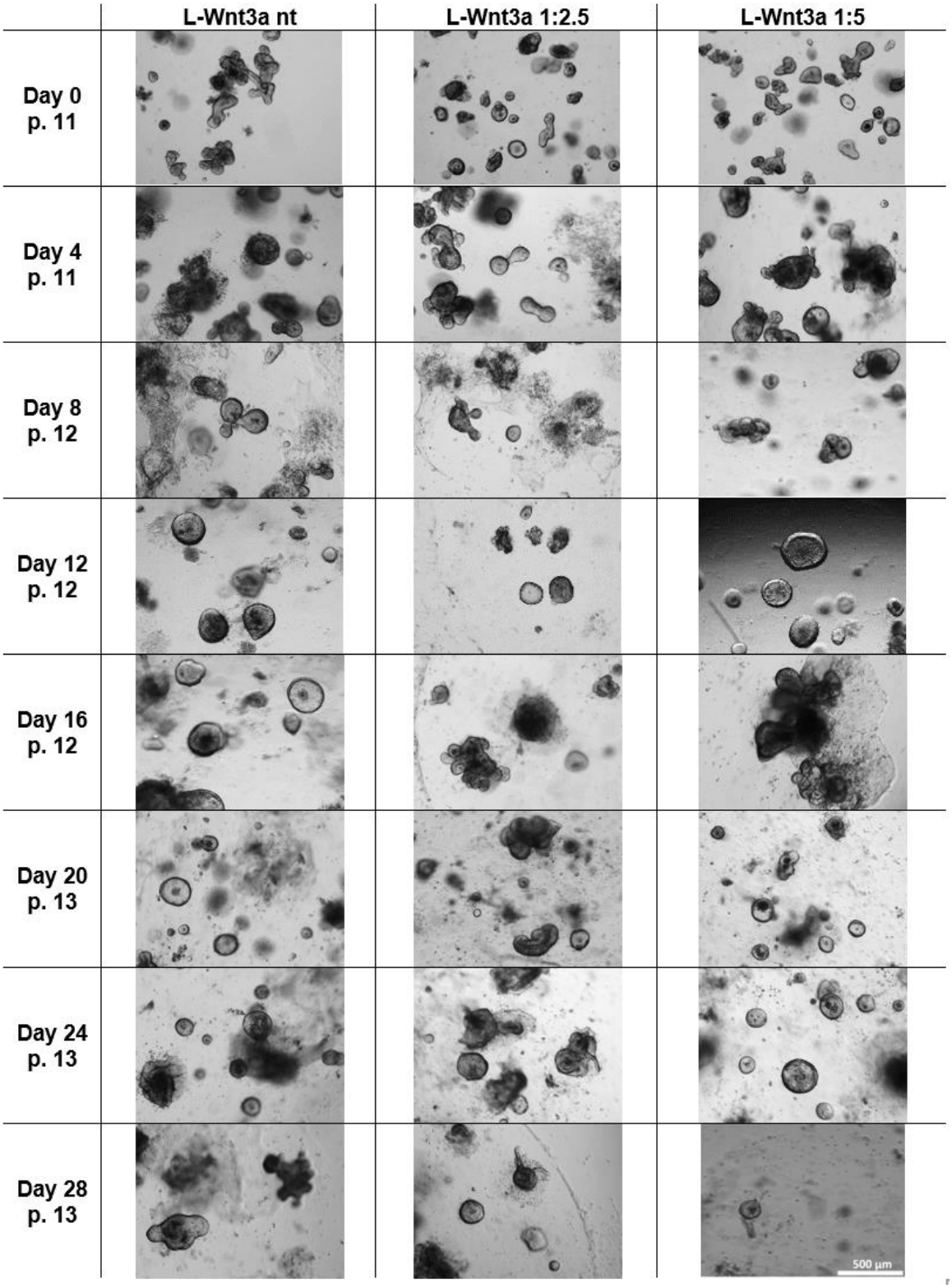

Figure 11: *Light microscopic images of colon organoids cultivated in c.m. produced by L-Wnt3a cells for 28 days; scale bar represents 500 µm. p = passage*

4. Discussion

Intestinal organoids are a promising *in vitro* model to study not only pathways but also mechanisms implicated in epithelial damage and repair. It is possible to generate organoids that are characterised by life-long self-renewal and multipotent differentiation potential from a single adult intestinal stem cell. Hence, this model is a cutting-edge tool regarding regenerative medicine (Blutt et al., 2019). In order to improve their relevance for clinical applications it was decided to make first steps regarding animal origin-free medium and supplements.

Results show that the transfection of HEK293T and L-Wnt3a cells with the GFP (green fluorescence protein) vector worked, suggesting that both cell lines are well suited for lipid-mediated transfection. Interestingly, HEK293T cells are more receptive to transfection than L-Wnt3a cells. The PCR revealed that the transfected cells genomically integrated the BII-CMV-AfmW3A plasmid. Due to the fact that only the GFP-transfected control was completely eliminated during the Puromycin selection (data not shown), we assumed that the transfected piggyBac vector was not only integrated in the genome but also successfully expressed by the selected cells. This assumption was made because the vector encodes Puromycin resistance in addition to Afamin-Wnt3a complex.

One major goal of this thesis was to elucidate whether the conditioned media obtained from the transfected cells can activate the canonical Wnt pathway as well as the conditioned medium obtained from cells cultivated in medium containing FBS. Surprisingly, the conditioned media obtained from the transfected and not-transfected cells did not activate the canonical Wnt pathway. Conditioned medium produced by L-Wnt3a nt was even activating the pathway slightly more than the others. It is possible that this happens because L-Wnt3a cells are already producing the “original” Wnt3a which is known for its ability to function to some extent even without FBS. So far one possible reason for this may be the sequence of the BII-CMV-AfmW3A plasmid which was used for the transfection. Afamin and Wnt3a are linked via a *Thosea asigna* virus 2A (T2A) self-cleaving peptide (GSGEGRGSLLTCGDVEENPGP). T2A is part of the class of 2A self-cleaving peptides which are found in viral families. Due to these peptides, it is possible to express multiple genes from the same mRNA by ribosomal skipping. The cleavage site is located between the residues of Glycine and Proline of the T2A peptide, leading to an Afamin which will have added some additional residues to the end, whereas the coding sequence from Wnt3a starts with the Proline (Fan, 2014). However, the exact mechanism of this cleavage remains unknown (“M. Ryan,” n.d.). Due to these additional residues, it may be

possible that the proteins are incorrectly folded. Hence, it may be likely that the proteins are ubiquitylated and degraded by the proteasome. The manuscript of the depositing laboratory is still in preparation and the plasmid has not been used in any other publication yet, which may support the hypothesis that there is a problem with correct protein expression.

This assumption could also explain why the gels of the SDS-PAGE showed bands at unexpected heights. Instead of one band at the height between 50 kDa and 75 kDa, two bands were expected from the conditioned media obtained from the transfected cells. The first one for Afamin should be at 70 kDa and the one for Wnt3a should be about 37 kDa. However, if the Afamin-Wnt3a complex is not secreted because of incorrect folding, it may be possible that there are no bands at the expected heights. As can be seen in Figure 8B the gel stained with Silver Stain shows that the control has a light band at the height of 37 kDa. This band cannot be seen on the gel stained with PageBlue™ Protein Staining Solution. That may be because the Silver Stain is more sensitive than the PageBlue™ Protein Staining Solution. This band shows that Wnt3a can be found in medium obtained from L-Wnt3a cells cultivated in medium containing FBS. As already mentioned, each conditioned medium contained proteins with a size between 50 kDa and 75 kDa. It is likely that these proteins are generally secreted proteins from L-Wnt3a and HEK293T cells. As described by Shin and colleagues different cytokines, growth factors and enzymes are secreted into the culture medium (Hathout, 2007). However, this mechanism depends on culture conditions and cell proliferation rate (Shin et al., 2019) and would explain why every conditioned medium shows one band at the height between 50 kDa and 75 kDa.

All canine colon organoids cultivated for 28 days were still viable. This is especially surprising, as the organoids treated with conditioned media obtained from not-transfected cells were cultivated in medium lacking Wnt3a. As already described by Fair and colleagues Wnt3a is inevitable for the regulation of self-renewal, proliferation and differentiation of organoids (Fair et al., 2018). It can be claimed that Paneth cells are producing the missing Wnt3a and hence their survival would be explained. However, Gelberg described that the intestine of dogs is missing Paneth cells (Gelberg, 2014). This result leads to the suggestion, that canine intestinal organoids may contain other cells compensating for Paneth cells. 2012 Rothenberg and colleagues described that in the murine colon, which is also lacking Paneth cells, cKit, a receptor for stem cell factors, marks cells which are not Paneth cells or goblet cells but something in between. This data suggests that these so called Paneth-like cells or deep crypt secretory cells could be replacing Paneth cells (Rothenberg et al., 2012). In 2019 a paper was

published by Chandra and colleagues which showed that Paneth-like cells are also present in the canine intestine (Chandra et al., 2019), possibly explaining the survival of the canine colon organoids treated with conditioned medium produced by not-transfected cells.

This hypothesis may also explain the viability of the organoids treated with the different conditioned media. Organoids treated with the conditioned media produced by HEK293T cells showed that the ones treated with the conditioned media produced by HEK293T nt had the lowest viability whereas the ones treated with the medium produced by HEK293T 1:2.5 had the highest. Interestingly, organoids treated with the conditioned medium obtained from L-Wnt3a cells had the highest viability when cultured in conditioned medium obtained from L-Wnt3a nt, and the lowest in conditioned medium produced by L-Wnt3a 1:2.5. The most viable organoids were seen when treating the cells with the conditioned medium obtained from cells cultured in medium containing FBS. Even though the organoids treated with the conditioned media obtained from HEK293T cells had the highest apoptosis after 24 h, considering apoptosis and necrosis, the cell death of the organoids treated with the conditioned media obtained from the not-transfected and transfected cells is approximately the same as the cell death of the control.

In order to optimize the production of conditioned media, follow-up experiments would be necessary. For example, to verify that all cells contain the BII-CMV-AfmW3A plasmid, several clones per cell line could have been selected and expanded. Hence, all cells would be derived from one single cell and would have the same genetic material. Meaning that if the plasmid integrates more than once into the genome of one cell, more Afamin-Wnt3a complex would be produced which would lead to a higher yield of the Afamin-Wnt3a complex. Also Reverse Transcription Quantitative PCR (RT-qPCR) would help to verify the expression of the genes of the vector. In order to verify the assumption that the Afamin-Wnt3a complex is not secreted further experiments would be necessary. For instance, Mass Spectrometry would provide information about the possible existence of the complex in the conditioned media. Also, Western blotting would help to provide more information, however no reliable Wnt3a antibodies are commercially available at the moment. Another possibility would be to add a tag (e.g. His) to Wnt3a within the plasmid and to enable purification of this tagged Wnt3a via affinity chromatography or Western blotting against the His-antigen.

This thesis shows that more time and research is necessary in order to produce a reasonable alternative for animal-origin free canine intestinal organoid culture. Until then, it is unavoidable

to use FBS but one should be aware of the meaning of this animal-derived product and the ethical questions raised regarding the blood collection.

5. Summary

Canine intestinal organoids are a great model to study human intestinal diseases because dogs develop similar gastrointestinal diseases as their owners. The cultivation of intestinal organoids needs the supplementation of Wnt3a to maintain the stemness of organoids.

Wnt3a is water-insoluble and needs the addition of foetal bovine serum containing carrier proteins building the water-soluble active Wnt3a complex. However, for the clinical application in regenerative medicine it is not possible to use the organoids cultivated in medium supplemented with animal-derived products. Mihara and colleagues discovered that Wnt3a complexed to Afamin is hydrophilic and biologically active.

This bachelor's thesis was aimed at comparing the serum-free conditioned media containing Wnt3a produced by L-Wnt3a and HEK293T cells transfect with a piggyBac vector constitutively expressing Afamin and Wnt3a and the Super piggyBac transposase expression vector for lipid-mediated transient transfection of the cells. Wnt signalling activation and the effect on organoid viability, apoptosis and necrosis were assessed to evaluate the functionality of produced media.

Results show that the integration of the plasmid coding for Afamin and Wnt3a was successful. However, it was not possible to activate the canonical Wnt pathway and to detect Wnt3a in the conditioned media. This leads to the assumption that the cells were unable to secret the Afamin-Wnt3a complex because of incorrect folding. Interestingly, all organoids survived a 28-day cultivation even though the medium was lacking Wnt3a. This could be explained by the presence of Paneth-like cells assuming the function of Wnt secretion *in vitro*.

Zusammenfassung

Hunde entwickeln ähnliche gastrointestinale Erkrankungen wie ihre Besitzer und daher stellen canine intestinale Organoide ein geeignetes Modell dar, auch humane intestinale Krankheiten zu untersuchen. Um diese Organoide kultivieren zu können, ist es notwendig, dem Nährmedium Wnt3a zuzusetzen.

Wnt3a ist ein wasserunlösliches Protein und kann nur durch Zusatz von fötalem Kälberserum und darin enthaltenen komplexbildenden Proteinen eine wasserlösliche aktive Form bilden. Für eine klinische Anwendung von Organoiden in der regenerativen Medizin ist allerdings die Verwendung von tierischen Produkten nicht zulässig. Miharas Arbeitsgruppe hat jedoch herausgefunden, dass durch eine Koexpression von Afamin und Wnt3a ein wasserlöslicher Komplex entsteht, der biologisch aktiv bleibt.

Das Ziel dieser Bachelorarbeit bestand darin, serumfreies Wnt3a-beinhaltendes konditioniertes Medium zweier Zelllinien (L-Wnt3a und HEK293T) herzustellen und zu vergleichen. Für die Synthese von Afamin und Wnt3a wurde ein piggyBac Vektor gemeinsam mit einem Super piggyBac Transposase-Expressionsvektor verwendet, der unterstützend für die Lipofektion herangezogen wurde. Die Wnt-Signalaktivierung und die Auswirkungen auf die Viabilität, Apoptose und Nekrose der Organoide wurden untersucht, um die Funktionalität der hergestellten Medien bewerten zu können.

Die Ergebnisse zeigen, dass die Integration des Plasmids gut funktioniert hat. Jedoch war es nicht möglich, Wnt3a Proteine in den konditionierten Medien nachzuweisen, um damit den kanonischen Wnt Signalweg zu aktivieren. Aufgrund dieser Ergebnisse kann vermutet werden, dass die Zellen nicht in der Lage waren, den Afamin-Wnt3a Komplex zu sekretieren, da wahrscheinlich das Protein aufgrund falscher Faltung zuvor abgebaut wurde. Interessanterweise blieben die kultivierten Organoide über den Beobachtungszeitraum von 28 Tagen, trotz nicht nachweisbaren Wnt3a, viabel. Diese Ergebnisse lassen deshalb vermuten, dass canine intestinale Organoide Paneth-ähnliche Zellen enthalten, die die Funktion der Wnt Sekretion *in vitro* übernehmen.

6. List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
A83-01	Alk4/5/7 inhibitor
APC	adenomatous polyposis coli
Arm	armadillo repeat domain
ASCs	adult stem cells
BMP4	bone morphogenic protein 4
c.m.	Conditioned Media
CBC	crypt base columnar
CBP/p300	cyclic AMP response element-binding protein
CNS	central nervous system
CK1	casein kinase1
CRD	cysteine-rich domain
CRISPR Cas9	Clustered regularly interspaced short palindromic repeats associated protein 9
DIX	Dishevelled-Axin
Dkk	Dickkopf
DPBS	Dulbecco's Phosphate Buffered Saline
Dvl	Dishevelled
ECM	extracellular matrix
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swam
ER	endoplasmic reticulum
EVI	Evenness interrupted
FBS	Foetal Bovine Serum
FGF-2	fibroblast growth factor-2
fw	forward
Fzd	Frizzled
GSK-3	glycogen synthase kinase 3

HBSS	Hanks' Balanced Salt Solution
HGF	hepatocyte growth factor
HSPG	heparan sulfate proteoglycans
IGF-1	insulin-like growth factor 1
ISC	intestinal stem cells
LAR II	Luciferase Assay Reagent II
LB	lysogeny broth
LGR5	Leucine-rich-repeat-containing G-protein-coupled receptor 5
LRP5 and -6	Leucine-rich repeat-containing G-protein coupled receptor 5 and 6
MAPK	mitogen-activated protein kinase
MMTV	mouse mammary tumour virus
nt	Not treated
PCR	Polymerase chain reaction
Pen-Strep	Penicillin-Streptomycin
PSCs	pluripotent stem cells
qPCR	Real Time Quantitative PCR
rv	reverse
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SFRPs	Secreted Frizzled-Related Proteins
SRB	Sulforhodamine B
T2A	thosea asigna virus 2A
TA	transit-amplifying
TCF/LEF	T cell factor/lymphoid enhancer factor
WG	Wingless
WLS	Wntless
WNT-1	wingless-type MMTV integration site family, member 1
Y-27632	ROCK inhibitor

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