Colitis-Associated Colorectal Cancer in Mice: *In vivo* Disease Imaging and Insight into the AOM/DSS Model

by

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Preface and Acknowledgements

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Abstract

Inflammatory bowel diseases as ulcerative colitis and Cohn's disease represent significant health problems in the western world. Colon cancer is a common consequence of those chronic inflammatory disorders, thereby it is essential to improve knowledge and research level of this area. AOM/DSS is a known and well-established chemically induced mice model of colitis associated cancer. In order to make research based on this model more efficient and reliable, improvements are desired.

In vivo imaging with the use of specific fluorescent probes is a very convenient and recently strongly developed method worth exploiting in monitoring tumors development. Furthermore, introduction of knockout model enabling in depth exploration of colon cancer mechanisms shades light on the behavior of the disease. Particularly, due to the importance of insulin receptor, as a key regulator of homeostasis, also involved in cancer pathology, it would be of high interest to explore it in terms of colon cancer. On the other hand, exploring physiology of the healthy colon is also essential in fighting this serious medical issue and culturing colon epithelium *ex vivo* gives a broad range of opportunities for this matter. 3D *in vitro* culture of colon crypts enables development of spherical organoids, imitating intestinal epithelium environment. In combination with mice of different genetic background, growing colon crypts raises a vast spectrum of exploration.

The used methodology was based on a combination of Azoxymethane (AOM) with dextran sulfate sodium (DSS) that allowed induction of colitis associated colon cancer mice model. Tumor development was confirmed with weights monitoring, colonoscopy and tumor counts. *In vivo* imaging system with the use of fluorescent probes was tested for imaging of tumors in AOM/DSS model. Two types of probes potent for emitting signal in near-infrared light were used: active IntegriSense 680 and activatable ProSense 750. Insulin receptor knock out mice were genotyped to confirm loss-of-function mutation. Colon crypts were taken from healthy mice donors and 3D matrigel-based culture was set up in order to induce organoids development and survival *in vitro*.

As a result, AOM/DSS model was successfully obtained which was confirmed with numerous tumors developed in distal part of the colon. *In vivo* imaging with IntegriSens 680 did not give significantly strong signal either *in vivo* or *ex vivo*. Activatable probe ProSense 750 however failed *in vivo* imaging of induced tumors, provided some specific distinguishable signal *ex vivo*. Colon crypts were successfully isolated from mouse donors. Culture was maintained for 9 days during which colonoids grown and developed into variously shaped spherical structures. AOM/DSS model resembles human colitis associated colon cancer. Although utilized fluorescent probes did not give expected results *in vivo,* ProSense 750 due to positive *ex vivo* measurement sheds a light on potential use and future optimization of that method.

List of Abbreviations

2D	Two Dimensional
3D	Three Dimensional
3Rs	Three Rs principles
AOM	Azoxymethane
APC	Antigen Presenting Cells
bp	Base Pair
CAC	Colitis-associated cancer
CRC	Colorectal Cancer
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats
CD	Crohn's Disease
DC	Dendritic Cells
DMH	1,2-dimethylhydrazine
DSS	Dextran Sulfate Sodium
IBD	Inflammatory Bowel Disease
IEC	Intestinal Epithelial Cells
IFN-γ	Interferon gamma
IL	Interleukin
INSR	Insulin Receptor
IP	Intraperitoneal
IV	Intravenous
IVIS	*In Vivo* Imaging System
KO	Knockout
MAM	Methylazoxymethanol
Th	T helper cell
TNF-α	Tumor Necrosis Factor alpha
Treg	T regulatory cell
UC	Ulcerative Colitis
WT	Wild Type

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1 Introduction

Colitis-associated colon cancer (CAC) is a serious complication in people suffering from inflammatory bowel diseases. There is a high demand for further research in this field in order to improve prevention, diagnosis and treatment of colorectal cancer. Many conventional methods for modeling this pathological condition are available, however there is much room for improvement and introduction of new techniques left.

One of the main objectives of this project was to establish the application of near infrared, *in vivo* imaging system (IVIS) in the lab, in order to image colon tumor progression in well-established chemical (AOM/DSS) model of CAC. To achieve that, specific fluorescent probes were used for *in vivo* imaging of murine colons. It is an alternative way to the more conventional colonoscopy and classic tumorcounting, which we also performed. In order to monitor and image colon tumors development, chemically induced model of CAC was induced in mice.

In addition, this project was aimed to confirm the obtaining of the insulin receptor knockout mouse via genetic fecombination method. This model of CAC will open an area of new unexploraed possibilities. IVIS will also be very helpful in knockout mice studies, enabling us to compare their inflammatory phenotype.

Furthermore, this work includes optimizing the method for three-dimensional (3D) *in-vitro* culturing of colon epithelium. This is a functional and more physiological like tool compared to conventional cell lines.

1.1 Colitis associated Colorectal Cancer and its pathogenesis

Colorectal cancer (CRC) is the third most common type of cancer worldwide. Its mortality rate classifies it on a fourth position. (Fitzmaurice et al., 2015). Chronic inflammation, aside from other triggers, is one of the main reasons for development of neoplasia in the colon. CAC is a type of CRC induced by inflammatory bowel diseases (IBD). Although inflammation induced cancer is considered as less frequent than those caused by familial-genetic inclinations or sporadic cause, it is a serious complication in IBD (Yang et al., 2014). Statistically, CAC is the reason for one in six IBD patient's death (Bernstein et al., 2001, Choi and Zelig, 1994). Depending on its duration, extend and severity, intestinal inflammation increases the risk of CAC occurrence (Rutter et al., 2004, Choi and Zelig, 1994).

The prevalence of inflammation-induced cancer as a development of dysplasia is a well-known established concept (Ullman and Itzkowitz, 2011, Morson and Pang, 1967). It has been proven that CRC expand in a bottom-up fashion (Barker et al., 2009, Preston et al., 2003). This model assumes the origin of the cancer cells at the bottom of the crypts, where intestinal stem cells are located, and their expansion towards colon lumen (Preston et al., 2003). Initially, chronic inflammation causes injuries, which leads to development of dysplasia proceeding to colorectal carcinoma (Radtke and Clevers, 2005, Terzic et al., 2010). Aberrant crypts are the first notable changes that can develop further to tumorigenic tissue. Fur-

thermore, through promotion and progression, adenocarcinoma develops with the following possibility of invasion, resulting in metastasis (Radtke and Clevers, 2005, Ullman and Itzkowitz, 2011).

1.1.1 **Inflammatory Bowel Diseases**

Ulcerative Colitis (UC) and Cohn's Disease (CD) are chronic inflammatory disorders characterized by an unfavorable mucosal immune response. These conditions are manifested by relapsing disease flares which in the long term can cause tissue damage and complications, one of which is CRC (Danese and Fiocchi, 2011, Baumgart and Sandborn). The pathogenesis of IBD is composed of many irritating factors such as anitbiotics, microorganisms, diet, stress or smoking which when accumulate, affect the intestinal mucosal integrity (Neurath, 2014). As a result of environmental triggers and unfortunate genetic background, the mucosal barrier might be disturbed. Such barrier-leakage can lead to a hypersensitivity and intolerance against commensal microbiota. Bacterial products leaking through impaired intestinal epithelial layers activate the underlying immune cells and recruite more. Consequently, this also activates representatives of innate immunity like dendritic cells (DC) and macrophages - key antibody presenting cells (APC). While active, they release spectrum of pro- and anti-inflammatory cytokines triggering further activation and differentiation of the T cells, a component of the adaptive immune response. Upregulation or imbalance in cytokines such as Tumor Necrosis Factor alpha (TNF-α), interleukin-6 (IL-6), IL-12, IL-18, IL-23 or IL-1β, produced by DC and macrophages influences the balance in T cells activity (Sanchez-Munoz et al., 2008). Affected by released cytokines, T regulatory (Treg) cells do not fulfill their role, which is harmonizing immune response by its inhibition and suppression of inflammation. Absence of appropriate regulation is complemented with overreaction of effector T cells. Eventually, cytokines produced by Treg, type 1 T helper cells (Th1), Th2 and Th17 contribute to intensify the imbalance in the immune defense (Papadakis and Targan, 2000). Excessive activation and reduced regulation of innate and adaptive immune cells leads to aberrant immune responses. It follows that the combination of above mentioned disturbances in the immune response, as a consequence of the imbalance in production of pro- and anti-inflammatory cytokines, contributes to chronic inflammation development and disease deterioration (Neurath, 2014). IBD share similar contribution factors such ass age of emerging, frequency of prevalence and symptoms. Apart from genetic predispositions, environmental factors such as smoking, stress, diet or infections also increase the risk of IBD (Irfan M. Hisamuddin, 2004). Diarrhea, weight loss, anemia, fatigue, abdominal cramps and fever are the typical symptoms for both UC and CD. Aside from the gastrointestinal tract, other organs and systems manifest ongoing inflammation as well. Beside those analogies, there are many differences in location, course and cytokine profile amongst IBD.

UC is a disease involving the mucosa of the colon (Baumgart and Sandborn, 2012). It can be described as a continuous, superficial inflammation, occurring from the rectum to the proximal colon (Neurath, 2014). In comparison with CD, a constant low-grade inflammation in UC patients also in remission suggests the reason for higher risk of developing CAC (Bjerrum et al., 2010, Olsen et al., 2009). T cells in UC seem to mimic Th2 cell cytokines production pattern, manifested among others by enhanced expression of IL-13 and IL-5 (Monteleone et al.,

2006). On the contrary, CD can include all layers of the intestinal wall and affect all parts of the gastrointestinal tract, most commonly appearing in the distal ileum and the colon. In this case, the cytokine pattern is more typical for a Th1 response, characterized by TNF-α and interferon gamma (IFN-γ) overexpression (Neurath, 2014, Monteleone et al., 2006).

1.1.2 Inflammatory Bowel Disease in Colorectal Cancer development

As stated previously, the active immune response during IBD promotes neoplastic development (Ullman and Itzkowitz, 2011) via mucosal injury and thus increased cell proliferation (Yang et al., 2009, Balkwill and Mantovani, 2001). CAC development is promoted by the upregulation of many cytokines, including TNF-α, IL-6 and IL-1 (Wang et al., 2009). That combined with oxidative stress, resident microflora and disrupted epithelial barrier contributes to cancer development (Yang et al., 2009, Ullman and Itzkowitz, 2011). Accumulation of reactive oxygen species produced by inflammatory cells leads to a condition called oxyradical overload (Itzkowitz and Yio, 2004). This state in combination with pro-inflammatory cytokines induces mutations and genetic instability. In this case, tumor promotion is facilitated by activation of pro-inflammatory genes, gene mutations and influencing methylation (Itzkowitz and Yio, 2004, Hussain et al., 2003). Specifically, gene alterations include those within oncogenes, tumor-suppressor genes and DNA repair mechanism genes, while genetic imbalance appears as aneuploidy and microsatellite, chromosome instability (Itzkowitz and Yio, 2004). Occurrence of gene modifications is even more favored by the intensified cellular turnover. In the inflamed mucosa, intestinal epithelial cells (IEC) are incited and controlled by cytokines in terms of proliferation, differentiation, regeneration and survival (Neurath, 2014, Su et al., 2013). This inflammatory environment enhances both epithelial proliferation and regeneration (Neurath, 2014).

As previously clarified, the main trigger of CAC is the chronic inflammation driven by the imbalance of the proinflammatory cytokine secretion. Furthermore, genetic modifications present in CAC differ from the ones identified in sporadic caused colon cancers (Feagins et al., 2009). Not only the influence of cytokines but also intestine characteristics makes its turnover so rapid. Constant renewal of epithelial layer is enabled by the presence of intestinal crypts filled at the bottom with intestinal stem cells (Okamoto and Watanabe, 2005). While cells present on epithelial layer in the lumen constantly shed, the stem cells at the crypts base proliferate and differentiate in the upwards direction introducing new, differentiated IEC (Humphries and Wright, 2008). The harmony between cell death and proliferation is a key feature of tumor development. This is strongly promoted by the imbalance of the microenvironmental cytokines stimulating tumorgrowth and angiogenesis in addition to silencing antitumor immune activity (Grivennikov and Karin, 2010).

1.2 Modeling and Exploration of Colorectal Cancer

As previously stated, colorectal cancer is one of the most common malignances, causing death of many patients worldwide. Hence, research and exploration that enables better understanding and improved diagnosis, treatment and preferably prevention is highly desired.

1.2.1 *In Vivo* Imaging System (IVIS)

Fluorescent imaging is a highly progressing field of study, especially in terms of *in vivo* research. The ability to non-invasively obtain qualitative visualization as well as quantitatively assess target of interest makes IVIS a very powerful and useful tool for cancer research (Ntziachristos, 2006). *In vivo* imaging enables tracking the development of disease in a very efficient way. Firstly, one can follow the progression of a medical condition within the same animal, providing reliable and comparable data (Jeffrey D. Peterson, 2012). Apart from that, another advantage is the reduction of used laboratory animals with the same amount of data obtained, which follows the principle of the three Rs (3Rs) (Wells, 2011).

The crucial feature of successful *in vivo* imaging is the use of the appropriate light that minimizes potential background and disturbances of the produced signal. Within the living organism, many tissues (ex. fur) and biological components (ex. hemoglobin – biological chromophore; lipids, water) exhibit ability to scatter the light or emit auto-fluorescence (biological fluorophores: elastin, collagen or food originating chlorophyll) (Ntziachristos, 2006, Hilderbrand and Weissleder, 2010). By keeping potential absorption coefficient of scattering molecules at the minimum level and omitting spectrum of emission of autofluorophores, one can maximize the value of the obtained signal. This can be provided by the use of near-infrared fluorescent (NIRF) probes (Hilderbrand and Weissleder, 2010). The use of spectral window between 600 - 800 nm enables the most representative signal, avoiding disrupting events. In principle, fluorescent probe designed to specifically detect and bind the target of interest is exposed to light of appropriate wavelength, which elicits emission of light at different wavelengths. Consequently, this signal is detected, imaged and measured, enabling further analysis (Brian Herman, 2015).

Another useful feature of fluorescent imaging is the constant development and improvement of existing and new probes. Specific targeting of particular components by engineered probes is called direct imaging and includes two types of probes (Ntziachristos, 2006). One of them is based on exploitation of activatable fluorochromes, which can only be detected if cleaved by an enzyme. In this case, an agent is made of a fluorochrome bound to its quencher and consequently maintained inactive. The phenomenon of quenching- absorption of energy disables the relevant fluorochrome to emit desired signal until probe reaches its 'destination' (Tung, 2004). For instance this can be an enzyme, known to be overexpressed in the investigated area. As the probe reaches environment of the enzyme abundance, the bound is cleaved. This releases the fluorochrome, bringing it to an active state and thus emiting fluorescence under treatment with the appropriate light (Ntziachristos, 2006, Tung, 2004). Examples of such enzymes are metalloproteinases (MMPs) and cathepsins, which both have been proven to be overexpressed in IBD and CRC (Ding et al., 2014, Hausmann et al., 2004, Makitalo et al., 2010).

Another type of probes are the active agents. These probes are simply fluorochromes attached to affinity ligands specifically binding certain molecules. They can be antibodies, small proteins or molecules that are antagonists of the target. One of the appropriate targets utilized in active probe design and overexpressed in cancer is integrin $\alpha_V\beta_3$, a marker of angiogenesis (Hilderbrand and Weissleder, 2010, Montet et al., 2006, Vonlaufen et al., 2001).

There are two types of planar imaging. As previously mentioned, the principle of fluorescent imaging is based on shedding a light beam on animals injected with a probe and further capture of emitted light with the use of charge-coupled device (CCD) camera equipped with appropriate filters.

One way of obtaining images with the use of fluorescence is epi-illumination. In this method, captured light produced by fluorescence is recorded at the same site as the source of the light, therefore it is called back-emitted light (Ntziachristos, 2006). This kind of imaging depends on the depth of the target and is suitable for more shallow surfaces, yielding two dimensional (2D), planar images (Vasilis, 2006). Even though it reflects intensity of fluorescence, it does not evaluate the depth of the signal.

Other way of fluorescent imaging is trans-illumination, a method by which 3D, quantitative measurements can be taken (Vasilis, 2006). This technique consists of shedding the light from the bottom of the animal and collecting emitted fluorescence on the other site. It is recommended for deeper tissues and requires more preparations of the animal prior to imaging. Trans-illumination provides information on geometry, depth and intensity enabling quantification of fluorescence source. In addition, in combination with computer tomography, it gives the possibility to orientate the point of interest in anatomical context (Ntziachristos, 2006, Vasilis, 2006).

In conclusion, exploiting alternatives in fluorescence raises a great potential to conventional cancer development monitoring

1.2.2 **Murine Models**

The current level of knowledge on CRC unquestionably results from the extensive research led by means of functional and valuable models (De Robertis et al., 2011, Karim and Huso, 2013). Essentially, any phase of CRC can be recreated, to a certain extend by means of *in vitro* and *in vivo* models. Despite recent development and improvement of *in vitro* models, in terms of complexity and yielding variable data, *in vivo* models remain irreplaceable (Katt et al., 2016). While in *vitro* research provides more isolated mechanistic insight, *in vivo* models enable us to approach a research question in a more complex network, providing a broader picture (Katt et al., 2016).

A known and well-established way of exploring carcinogenesis of the colon is the use of rodent models. Undoubtedly, rodent models of colon cancer have their strong advantages over other available examples. Namely, the pathogenesis obtained in those models almost fully outlines the one in human CRC. Moreover, these models are highly reproducible and can be applied in animals with various genotypes and genetic background (Rosenberg et al., 2009). Mice are highly beneficial and applicable rodents for modeling CRC. A wide range of recombinant, transgenic mice, whose genetic background is well known, can be used. Additionally, mice models deliver high yield of tumors including adenoma-adenocarcinoma transformation (Rosenberg et al., 2009). There are a few ways of obtaining rodent models of CRC and each type has its own advantages and restrictions. One of them is a tumor implantation model, obtained by injection of tumor cells: subcutaneous, intravenous, into spleen or liver. By grafting

tumor cells from human (xenografts) or other animals of same species (auto-, allografts), into immunodeficient animals, tumor development and further analysis become possible. Introduction of orthotropic transplantation enabled monitoring tumor growth and metastasis, which becomes an advantage of that model over others lacking invasive and metastatic phenotype (Karim and Huso, 2013). Another advantage is the opportunity to introduce genetic modifications into grafted cancer cells, for example green fluorescence protein or luciferase, which simplifies further examination (De Robertis et al., 2011, Garofalo et al., 1993).

A different type of CRC model is the genetically modified animal as for example ΔAPC-mice (De Robertis et al., 2011). This model enables monitoring of initiation, promotion and progression of tumor development but as mentioned before, it is not always able to reflect metastasis stage. Mutations-carrying mice are generated, leading to development of intestinal neoplasia. Targeted mutations include genes known to be involved in CRC development and those which while altered favor pro-carcinogenic environment, for example mutations in β-catenin or various tissue-specific mutations of APC (De Robertis et al., 2011).

Last, but not least is the chemically induced tumor model. Among different available chemical substances acting as carcinogens and therefore eliciting neoplastic histogenesis, azoxymethane (AOM) offers a lot of advantages, which makes it the most commonly used one (Rosenberg et al., 2009). AOM is an indirect-acting, organotropic colon carcinogen metabolite widely applied in the past as 1,2-dimethylhydrazine (DMH). As an indirect agent, AOM needs to be activated by hydroxylation to reactive methylazoxymethanol (MAM). Furthermore, the methylation of guanine at 06-position lead by this metabolite is considered as exact pro-mutagenic activity, favoring carcinogenesis (Delker et al., 1998).

When comparing AOM to other alternative substances it is relatively cheap and easy to administrate, providing high efficiency and reproducibility (De Robertis et al., 2011, Neufert et al., 2007). AOM in combination with an inflammatory agent like dextran sodium sulphate (DSS) enables an easy and effective way of obtaining a CAC model. DSS causes severe injury in the epithelium of the colon. Injecting a mouse with AOM and further treatment with DSS in drinking water enables us to obtain tumors which are histologically resembling CAC in human, making it a valuable and reliable model for cancer research (Tanaka et al., 2003a, Neufert et al., 2007). Different protocols for the AOM/DSS model have been suggested, using various mice strains to mimic CAC. As each mice strain shows different susceptibility towards various carcinogens, it has been proven AOM/DSS combination is an appropriate choice for C57Bl/6 strain (Diwan and Blackman, 1980, Rosenberg et al., 2009)

1.2.3 **Murine knockout models and the INSR in CRC**

The cancer cell is a mutated cell where growth, division and life span are altered due to combination of single mutations, influencing these complex processes (Hanahan and Weinberg, 2000). The understanding of underlying molecular and cellular processes and their dysfunction in the context of cancer research is crucial for development of successful treatments. In order to explain the role of particular pathways or processes in the cell, introduction of loss-of-function studies can be done (Walrath et al., 2010). With constantly developing technology, many methods for introducing gene knockouts are available. CRISPR-Cas9 (Clustered

Regularly Interspaced Short Palindromic Repeats) is a promising and powerful, recently discovered and optimized technique for targeted genome editing. CRISPR originates from the bacterial adaptive immune system; where RNA guided nucleases introduce double stranded DNA cleavage. By this, silencing of a particular gene is possible via endonuclease activity, cutting out sequence of interest (Sander and Joung, 2014). Other way of achieving knockout mice is by means of site-specific recombinase technique enabling introduction of DNA alterations as deletions or insertions in desired genes (Branda and Dymecki, 2004). In that case, Cre-lox recombination is a widely used method employing the P1 bacteriophage derived genetic recombinant enzyme (Cre recombinase) to recognize and cut at a specific site within the gene (Nagy, 2000). Essentially, the element of interest, that is supposed to be altered is flanked by sequences called lox P sites, which are the recognition spots for Cre recombinase (Hamilton and Abremski, 1984). Lox P sites are 34 bp, palindromic constructs of following sequence: ATAACTTCGTATA - NNNTANNN – TATACGAAGTTAT (N stands for wariable bp) (Nagy, 2000). Additionally an introduced factor for this technique can be tissue specific recombinase, where a promoter can control the expression only in particular tissues or organs. As a consequence, the recombinase can be expressed in a defined part of the organism (el Marjou et al., 2004, Feil et al., 2009). Mouse bearing tissue specific Cre recombinase crossbred with mouse carrying loxP sites, flanking part of the gene that is supposed to be modified, inactivates the gene of interest. (Hamilton and Abremski, 1984).

As mentioned before, identifying key elements in cancer development and function is of high clinical importance. It has been demonstrated that the insulin receptor (INSR) is overexpressed in dysplastic mucosa of UC patients. This finding might suggest that INSR is involved in colorectal inflammation and tumor genesis (Bjerrum et al., 2014). INSR is involved in regulation of different processes, especially of the cell metabolism (Lee and Pilch, 1994). Moreover, it is also known that cancer cells are characterized by modified energy metabolism (Vander Heiden et al., 2009).

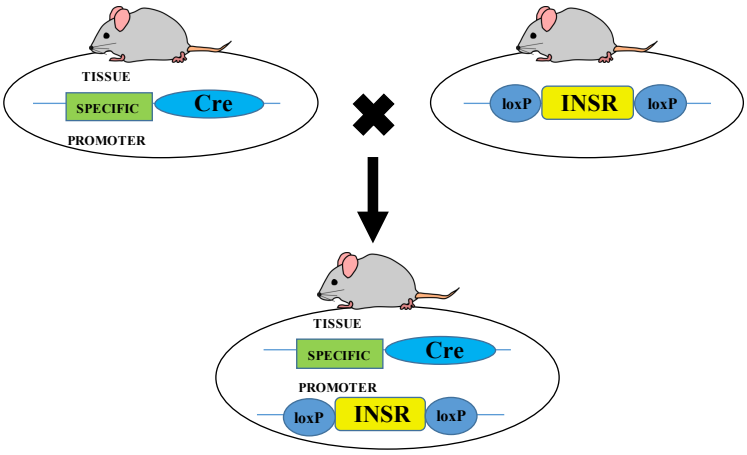

Figure 1.2.3.1 Scheme of Cre-lox recombinantion intending to deactivate INSR.

All the described arguments lead to the conclusion that an understanding of the INSR involvement in CRC is an important aspect worth exploring. This can be possible by introduc-

tion of intestinal INSR knockout mice by means of the described Cre-lox recombination technique. This knockout model will enable us to elucidate the importance of the INSR in CAC.

1.2.4 **Organoids**

As previously stated, it is crucial to study how CRC develops in order to be able to improve treatment and prevention. There are different ways of investigating underlying mechanisms of disease. Animal model studies are one of the most valuable sources of information about human diseases and are often an unavoidable step in translating knowledge of discoveries to the clinic. Animal models provide physiological relevant information in contrast to *in vitro* research

Even though using animals in research has many advantages there are meaningful reasons for replacement of that exploration tool. Some of them are expensiveness, long duration of technique performance, and ethical concerns (Arora et al., 2011). In other words, it is crucial to also develop substitutes of investigation.

Widely used, alternative ways for animal research are *in vitro* cultures. This technique is much less expensive, simpler, replicable method, yielding high throughput. Besides, it allows fulfilling 3Rs principle of animal use reduction (Gruber and Hartung, 2004). Despite those advantages, it is often the case, that *in vitro* techniques are insufficient in translating overall picture of the problem. Additionally, in terms of colon pathogenesis research, colon crypt cells are impossible to grow as they cannot sustain in a monolayer culture (Tan et al., 2015). Furthermore, colon cancer cell lines do not represent an accurate picture of the real microenvironment present in the intestinal epithelium. For these reasons any conclusions coming from this kind of research have to be driven with caution

Cancer is a condition of aberrant stem cell differentiation, suggesting presence of cancer type stem cells. Possibility to undergo all required modifications to become a cancerous cell is stated to be more likely to happen to stem cell than differentiated ones (Radtke and Clevers, 2005). That is why research on intestinal stem cells, their role and function in physiological and pathological cases is very important in revealing underlying mechanisms. Accordingly, especially in research on CRC and colon epithelium, introduction and validation of 3D culture system is so important.

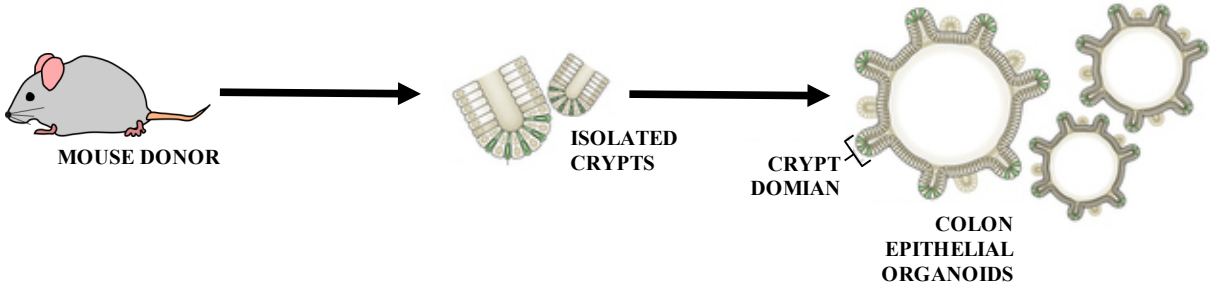

Figure 1.2.4.1 Scheme of colon crypts isolation and organoids development in *ex vivo* culture. Image partially adapted from (Barker, 2014)

The concept is to collect colon from a donor mouse, isolate crypts and by providing the adequate environment, enable development of spherical crypts formations called organoids (Shamir and Ewald, 2014). They are 3D, multicellular structures, containing key features of intestinal epithelium including crypt like structures. By means of colonic stem cells activity, colonoids (organoids originating from colon) can be grown. In order to do that, special conditions have to be fulfilled. Providing 3D-embedded culture environment and supplementation of media with appropriate growth factors are one of the crucial requirements in order to keep crypts alive and developing (Shamir and Ewald, 2014). This conventional tool gives possibility for broad range of applications in functionally and physiologically relevant model systems. Eventually, analysis on molecular and cellular basis, also in real time becomes possible and mechanistical insight can be achieved. By growing this context-specific culture, one gains opportunity to explore mechanisms within both physiological and pathological intestinal epithelium, as proven by Sato and colleges (2011) who cultured colon adenocarcinoma organoids.

2 Materials and methods

2.1 Mouse Model

C57BL/6 mice were obtained from Jackson Laboratory (Sacramento, California, US). B6.129S4(FVB)-*Insr*^{tm1Khn}/J and B6. Cg-Tg(Vil-cre)997Gum/J were used to generate insulin receptor knock-out mice with the use of Cre-Lox recombination technology. These mice were bred under specific-pathogen-free conditions. All animal studies were approved and licensed by the Danish Council of Animal Experimentation.

2.1.1 **AOM/DSS model**

Wild type (WT) C57BL/6, female, age-matched mice were given intraperitoneal (IP) injection of the carcinogen AOM (Sigma-Aldrich, St. Louis, Missouri, US) at a dose of 7.4 mg/kg, diluted with phosphate buffer saline (PBS without Ca, Mg). Mice were fed ad libitum with a 12 h light/dark cycle. During the following 2 weeks the animals were kept in class 2 GMO laboratory. Thereafter, DSS (Sigma-Aldrich) dissolved in Milli-Q water at a concentration of 3% (w/v) was given to the animals in their drinking water for 1 week, following 2 weeks of remission with clean water. This was repeated 2 times. Monitoring animal's weight, evaluation of diarrhea and rectal bleeding enabled to assess colitis severity. After the whole treatment session, animals were sacrificed by cervical dislocation and colons were collected for tumor counting and assessment of localization.

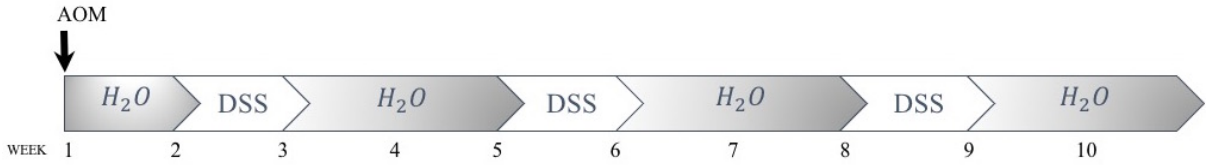

Figure 2.1. Scheme of AOM/DSS treatment.

2.1.2 **Colonoscopy**

With help from one of the members of the research group, colonoscopy examinations of AOM/DSS treated mice were performed. In order to do so, the animals were anaesthetized with isoflurane (Aerrane Isoflurane, Baxter International, Deerfield, Illinois, US) and examined with the use of endoscopy equipment (Endoscope xenon 175, telecam SL, Karl Storz GmbH & Co KG, Tuttlingen, Germany).

2.2 IVIS

Prior to imaging, obtained AOM/DSS animal mice models were fed with purified chow free diet (Research Diets, NJ, US) for one week to minimize autofluorescense from the chlorophyll. To enable conceivable and reliable imaging animals were shaved (Contura Trimmer, Wella Professionals, Geneva, Switzerland) and depilated from remaining fur (Veet Hair removal cream, Reckitt Benckiser, Slough, UK) on the whole abdominal part, one day before

imaging. This was done in order to minimise scattering of the light, especially strong in terms of dark fur. During the hair removal procedure animals were anaesthetized with isoflurane (Aerrane Isoflurane, Baxter Internationals). After depilating, skin was thoroughly cleaned with water and treated with pure lanolin ointment (Purelan 100, Medela AG, Eching, Germany) to avert irritation.

Two types of fluorescent probes were used in order to capture developed colorectal tumors. Activatable ProSense 750 EX and active IntegriSense 680 (PerkinElmer, Boston, US) fluorescent *in vivo* imaging agents were reconstituted in PBS and injected intravenously, 24 h before imaging, using recommended dose of 2 nmol/100 µL. Appropriate controls were designed in order to obtain reliable picture of background signal and qualify signal of interest.

In total 8 mice were imaged in the following manner (Fig. 2.2): two AOM/DSS treated model mice and one non treated control, injected with ProSense 750 EX; two AOM/DSS treated and one untreated control, injected with IntegriSense 680; one AOM/DSS treated mouse with no probe and one untreated mouse without probe. Imaging was performed with the use of IVIS Spectrum CT (PerkinElmer). Animals were anaesthetised with isoflurane (Baxter Internationals) while the pictures were taken. After *in vivo* imaging, while anaesthetized, mice were sacrificed by cervical dislocation and colons were dissected for *ex vivo* imaging.

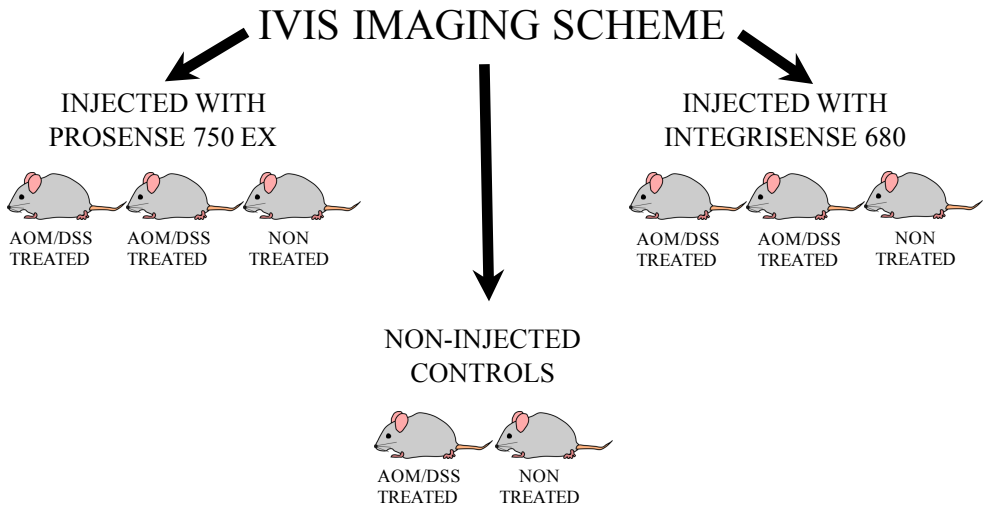

Figure 2.2 Scheme of IVIS imaging. Three mice were injected with ProSense 750 EX and three mice were injected with IntegriSense 680, in each group one untreated contol mouse was included. Additionally two control mice were imaged without being injected with any probe, one AOM/DSS treated and one non-treated. In total eight mice were imaged in IVIS experiment.

2.3 Insulin Receptor knockout model

2.3.1 Genotyping

2.3.1.1 DNA isolation

INSR knockout mice were genotyped with genoming DNA isolation from tail samples using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Mice tail samples were taken and DNA isolation was performed using the kit according to the manufacturers instructions. Obtained purified DNA was amplified in a PCR reaction. The reaction volume was 50 µl (49 µl of PCR master mix prepared according to polymerase manufacturer's instructions**,** 1 µl template sample) and 36 cycles were programmed (DNA Engine Dyad cycler,) with the use of Dream Taq DNA polymerase (Thermo Fisher Scientific, Waltham, USA). The PCR cycling parameters were as follows: denaturate at 94°C for 45 s, anneal at 58°C for 45 s and extend at 72°C for 1 min. Each DNA template sample was amplified separately, with two primer sets acquired from Jackson Laboratory. The first set of primers identified loxP sites on 4^{th} exon, in gene coding insulin receptor; Primer Sense: 5'-GGG GCA GTG AGT ATT TTG GA-3', Primer AntiSense: 5'-TGG CCG TGA AAG TTA AGA GG-3'. The second set of primers identified villin tissue specific Cre-recombinase; Primer Sense: 5'-CAT GTC CAT CAG GTT CTT GC-3', Primer AntiSense: 5'-TTC TCC TCT AGG CTC GTC CA-3'.

2.3.1.2 Agarose electrophoresis

Amplified DNA was separated in agarose electrophoresis at 120V for 60 min (VWR Power supplies, Radnor, Pennsylvania, US; The Centipede Wide-format Electrophoresis system, Gel company, San Frnacisco, US) in 3% agarose gel with etidium bromide (MultiPurpose Agarose, Tebu-bio, Le Perray-en-Yvelines, France; 1% TAE Buffer, Thermo Fisher Scientific; Ethidium bromide Solution 0.07%, AppliChem Panreac, Darmstadt, Germany) loading 30 µl of a sample (25 µl of a sample, 5 µl of dye (6x DNA loading Dye, Thermo Scientific)) and 10 µl of a ladder (Gene Ruler 50bp, Thermo Scientific).

2.4 Mouse Colon Organoids Culturing

2.4.1 Colon crypts isolation

The distal part of a colon (excluding cecum) was taken from mouse donor. The colon was cleaned from residues of other tissues and cut open (longitudinal cut) with the use of dissection scissors and a scalpel. Specimen was further rinsed with cold PBS, sliced in about 2 mm pieces and placed in cold PBS on ice. The colon pieces were washed by adding 15 ml of cold PBS and pipetting the supernatant away after intestinal pieces settled by gravity. This step was repeated about 15 times, until supernatant was clear. Intestinal pieces were incubated in chelation buffer (recipe taken from (Tan et al., 2015); PBS with EDTA 3mM and DTT 0.05mM) for 60 min. Supernatant was removed. Procedure of washing with 10ml of cold PBS, as described above, was repeated twice. After the second wash, when removing supernatant, about 2 ml were left in the tube. Remaining PBS with intestinal pieces was gently shaken. The supernatant was collected and evaluated in terms of crypts presence with the use of microscope (Evos Imaging System, AMG, Bothell, US). Procedure was repeated up to 8 frac-

tions and those rich in crypts were pooled together and centrifuged at 290 x g for 5 min in 5°C. Pellet was retained.

2.4.2 **Colon crypts culture set up**

Pellet obtained from colon crypts isolation step was further treated according to Stemcell Technologies protocol for Intestinal Epithelial Organoid Culture with Intesti Cult Organoid Medium (Mouse) in order to set up 3D culture system for organoids growth (Stemcell Technologies, Vancouver, Canada). Organoid growth medium (OGM), supplied by the manufacturer was complemented with 0.1 μg/ml Wnt3 ligand (rhWnt-3a, recombinant human CHO cell-derived, R&D Systems, Minneapolis, US) in order to enable colonic crypts to survive in the culture and develop organoids. OGM mixed with Matrigel (Growth Factor Reduced (GFR), Basement Membrane Matrix, Corning, New York, US) enabled creation of spherical domes at the bottom of each well of 24 Well Clear Not Treated Plate (Corning, New York, US). Domed cultures were supplied with additional OGM, filling the well and covering gel surface. Plate containing the organoid cultures was kept in an incubator (CO_2, Water Jacketed Incubator, Forma Scientific, Thermo Scientific) at 37°C and 5% CO_2.

Colonoid growth was monitored with the use of microscope (EVOS, Cell Imaging System, AMG, Thermo Fisher) with build in digital camera, enabling visual measure taking pictures of colon crypts development. According to supplier recommendations, growth media was changed 3 times per week. Organoids were kept in culture for nine days.

3 Results

3.1 AOM/DSS mice model of CAC

Mice weight loss has enable us to monitor colitis severity. According to Figure 3.1.1 mice treated with AOM/DSS experienced weight loss after the DSS cycles, which indicated inflammation and colitis development. Weight curve of untreated controls illustrates rather steady increase in weight, Inflammation was additionally confirmed by the presence of diarrhea, during DSS treatment.

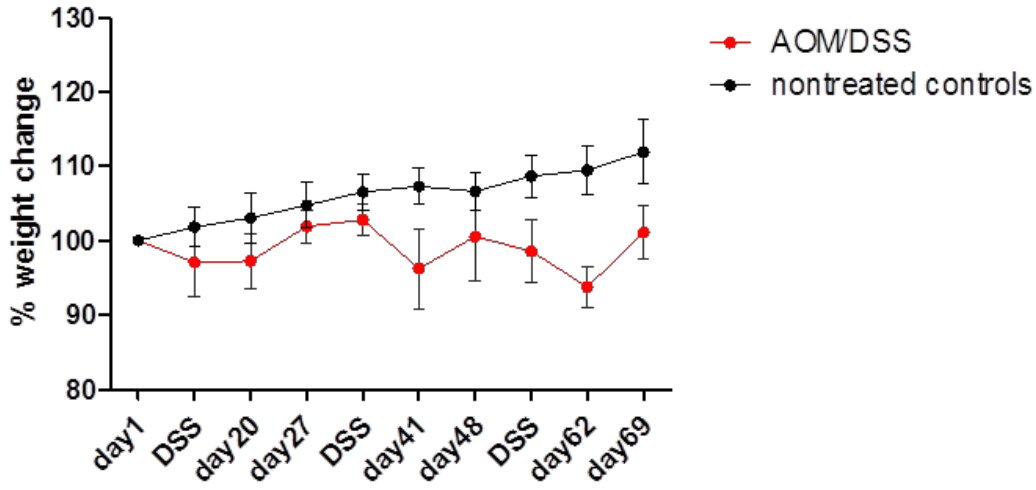

Figure 3.1.1 Weight curve. Mouse weights relative to the baseline (day 0). The black curve indicates control group – non-treated mice; n= 12. The red curve presents weights of AOM/DSS treated mice; n= 5 (n= number of mice in each group).

Numerous tumors were found in the middle and distal colon of mice treated with AOM/DSS. The average total number of tumors per mouse was 7 (Fig. 3.1.2). As seen in Figure 3.1.3, highest tumor burden was detected in distal part of the colon. It should be noted that the majority of the tumors were those of < 2mm diameter (Fig. 3.1.2).

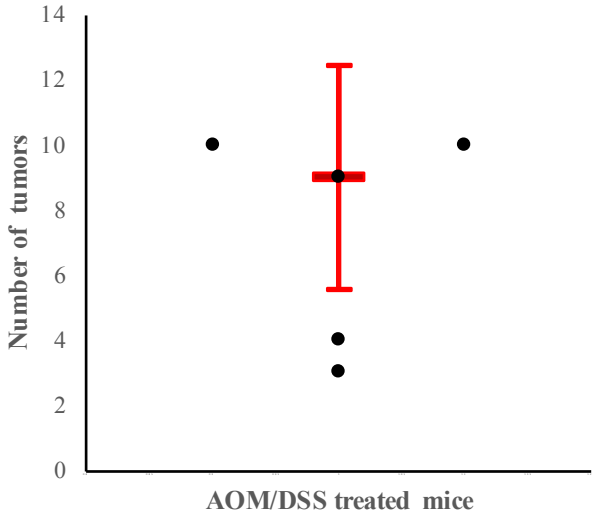

Figure 3.1.2 Total tumor counts. Each dot represents total tumor count for a specific mouse, treated with AOM/DSS. Graph includes median and standard deviation of total tumor counts.

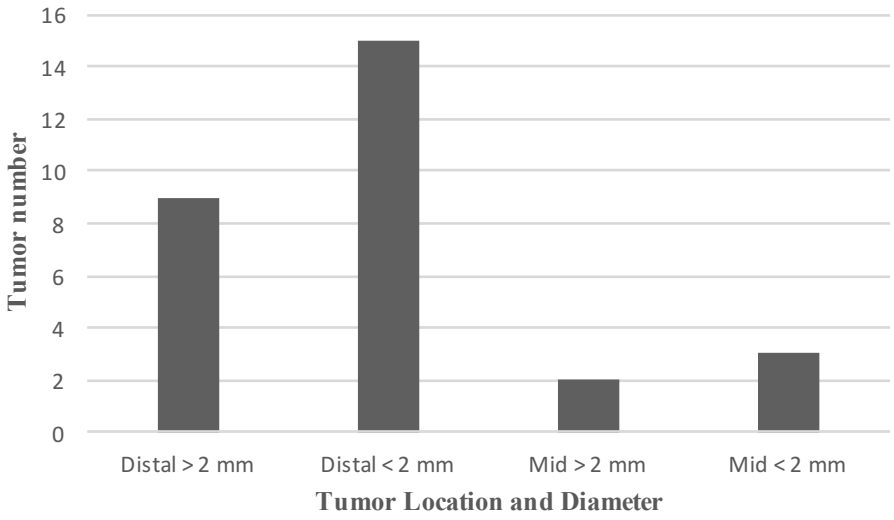

Figure 3.1.3 Size and distribution of the tumors. Representative distribution of the total number of AOM/DSS treated mice tumors, including tumors diameter. It can be noted that the majority of the tumors was detected in distal area and the greater part either in distal or mid colon were the ones of diameter < 2 mm.

The dissection of colons seen in Figure 3.1.2 presents localization of the tumors, mostly present in distal and middle area. Different sizes of tumors can be seen in pictures A and B of Figure 3.1.2. Colonoscopy examination performed prior to sacrifice, revealed tumor presence in the AOM/DSS treated mice (Fig. 3.1.3). Tendency for distal localization of tumors can also be noted via this examination (Fig. 3.1.3 B). Picture I in Figure 3.1.3 B illustrates inflamed mucosa of the colon while following images II-IV present tumors protruding into colon lumen. Image III and IV illustrate how strongly tumors interfere with colon almost completely obstructing its lumen.

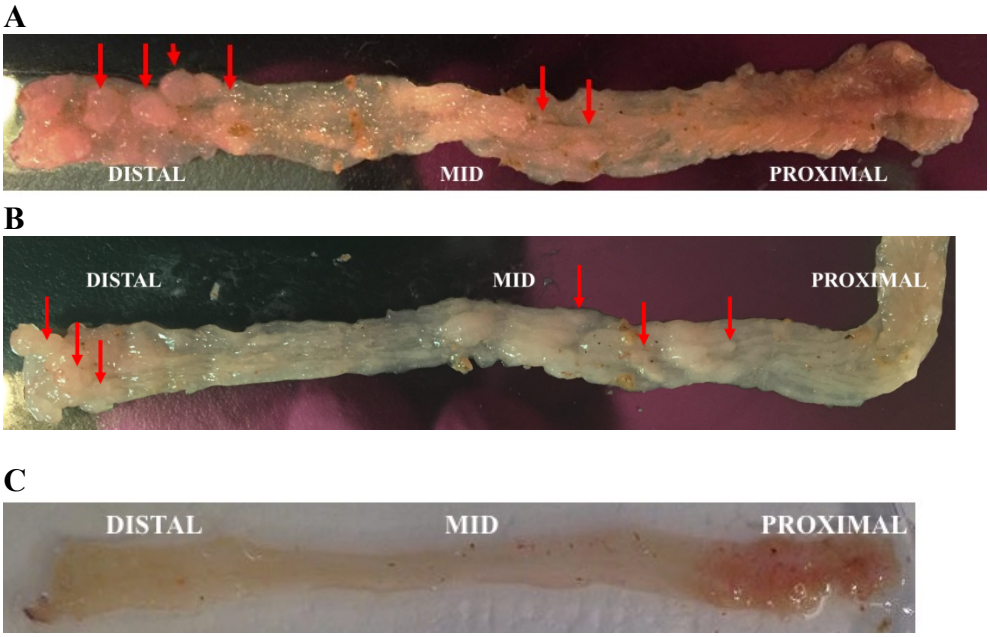

Figure 2.1.2 Longitudal speciemens of mouse colons. (A), (B) AOM/DSS treated mice colons. Arrows indicate tumors. (C) Untreated, control mouse colon.

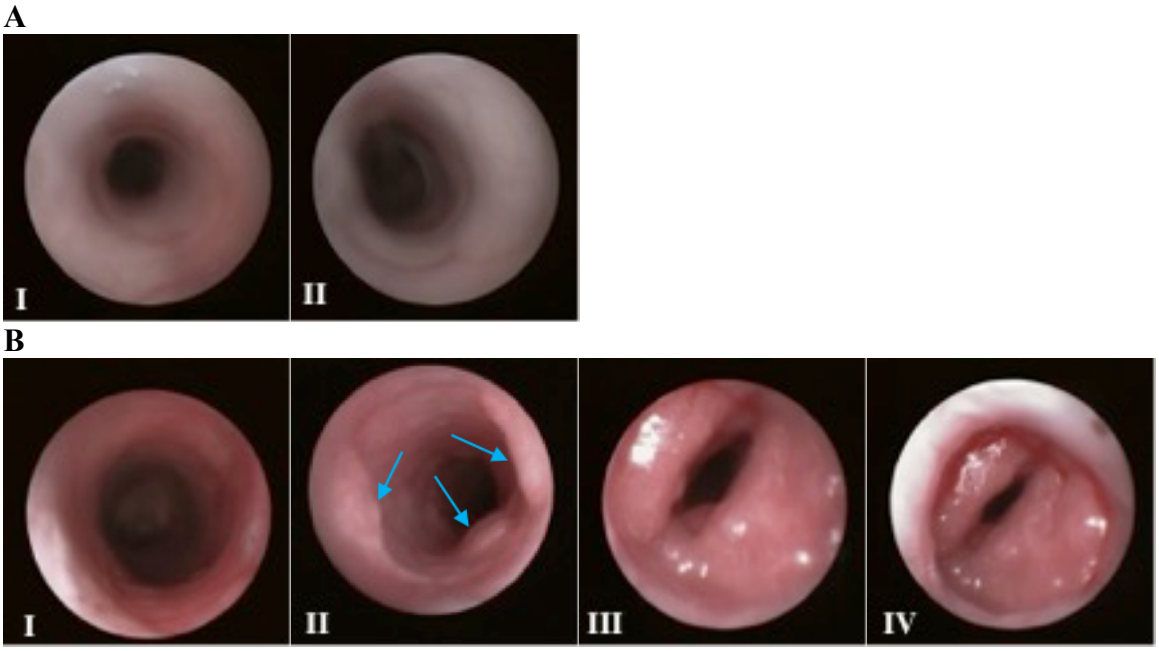

Figure 3.1.3 Murine endoscopy. Colonoscopic view in murine colons. (A) I, II - pictures of control, healthy colon of untreated mouse. (B) View of AOM/DSS treated mice colons; I - image of inflamed colon; II, III, IV - images of colons with tumors obstructing the lumen of the colon; II – blue arrows indicate tumors. Picture III, IV – tumors severely obstruct colon lumen. Image III and IV present more distal parts while II and I are located closer to mid part of the colon.

3.3 IVIS

Mice utilized in this experiment were chemically induced in order to develop colon tumors, modeling colitis-associated cancer. To that end animals were monitored in terms of colitis severity in the same way as previously described. Figure 3.2 illustrates weight curves of AOM/DSS treated mice, destined for IVIS experiment. It can be seen, each mice reacts slightly different to applied therapy, with general trend to loose weight after each DSS cycle.

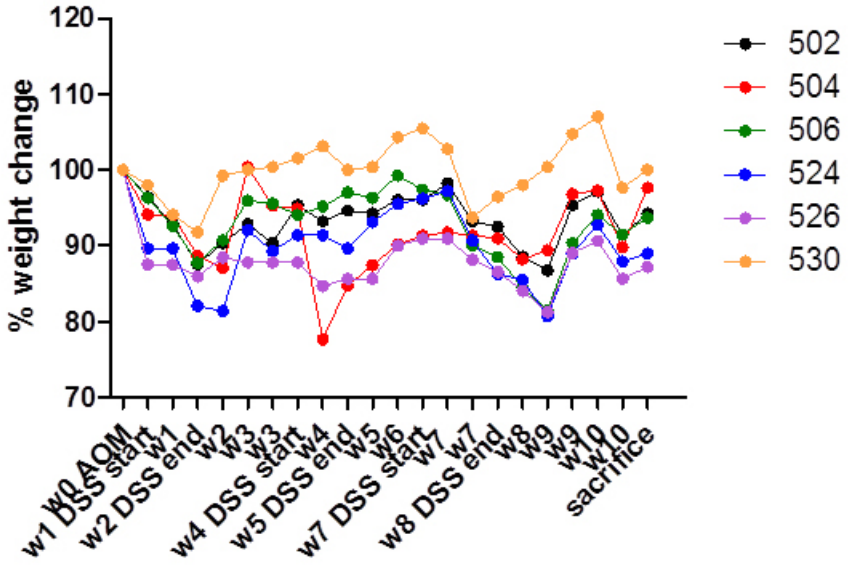

Figure 3.2. **Relative weights of AOM/DSS treated mice**. Each number (502-530) indicates each mouse.

3.3.1 ProSense 750 EX

In vivo NIRF imaging, with the use of activable probe ProSense 750 EX yielded two-dimensional (2D) pictures of mice. Pictures of different color intensity were taken in order to asses the specificity of obtained signals (Fig. 3.2.1.1 and 3.2.1.2). As seen in Figure 3.2.1.1 and 3.2.1.2, mouse without injected probe but AOM/DSS treated, gave very little background signal (mice A and D). Mouse treated with AOM/DSS expected to develop tumors and injected with ProSense probe (mice B in Fig. 3.2.1.1) gave stronger, specifically located signal. However is was detected as a signal not specific for tumors. Mouse E from Figure 3.2.1.2, having the same features as mice B, indicated the importance of intravenous (IV) injection of the probe. After unsuccessfull IV injection, an intreperiotenal (IP) injection was performed, yielding weak signal from the abdomen and strong signal from the tail. Mouse C (Fig. 3.2.1.1) and F (Fig. 3.2.1.2) indicate presence of unspecific signal, as both animals although injected with fluorescent probe, were non-treated with AOM/DSS.

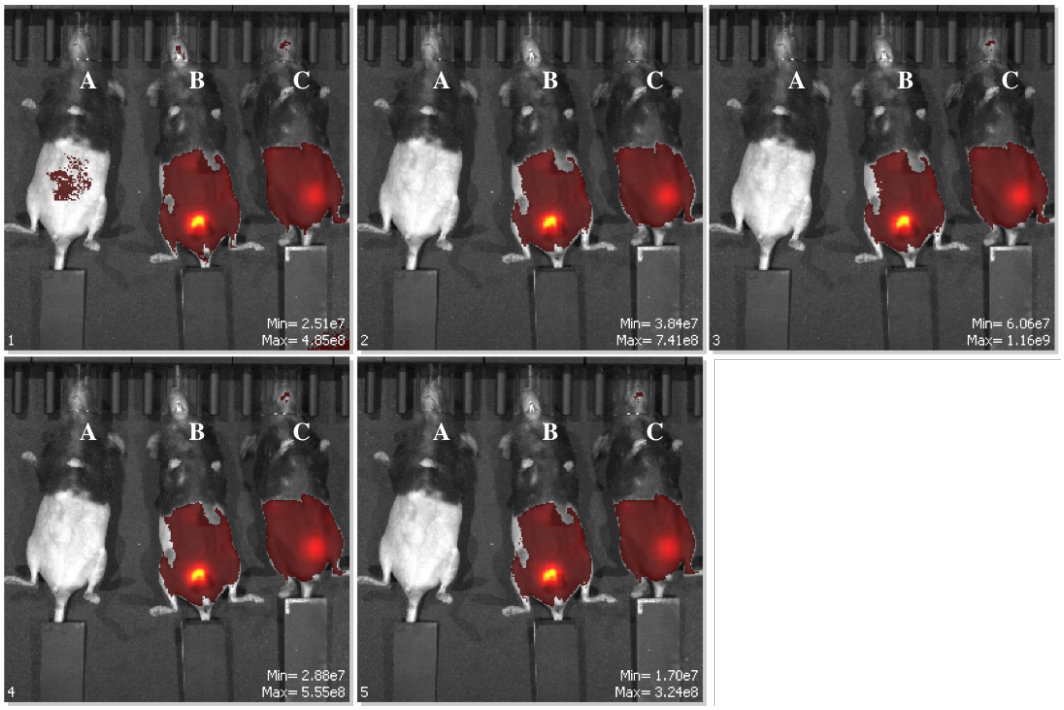

Figure 3.2.1.1 *In vivo* NIRF images of mice injected with ProSense 750 EX including controls. All pictures present the same animals, each image has different intensity of color scale; A – AOM/DSS treated, no probe (auto-fluorescence control); B – AOM/DSS treated, ProSense 750 EX injected; C – untreated, ProSense 750 EX injected (non-specific signal control).

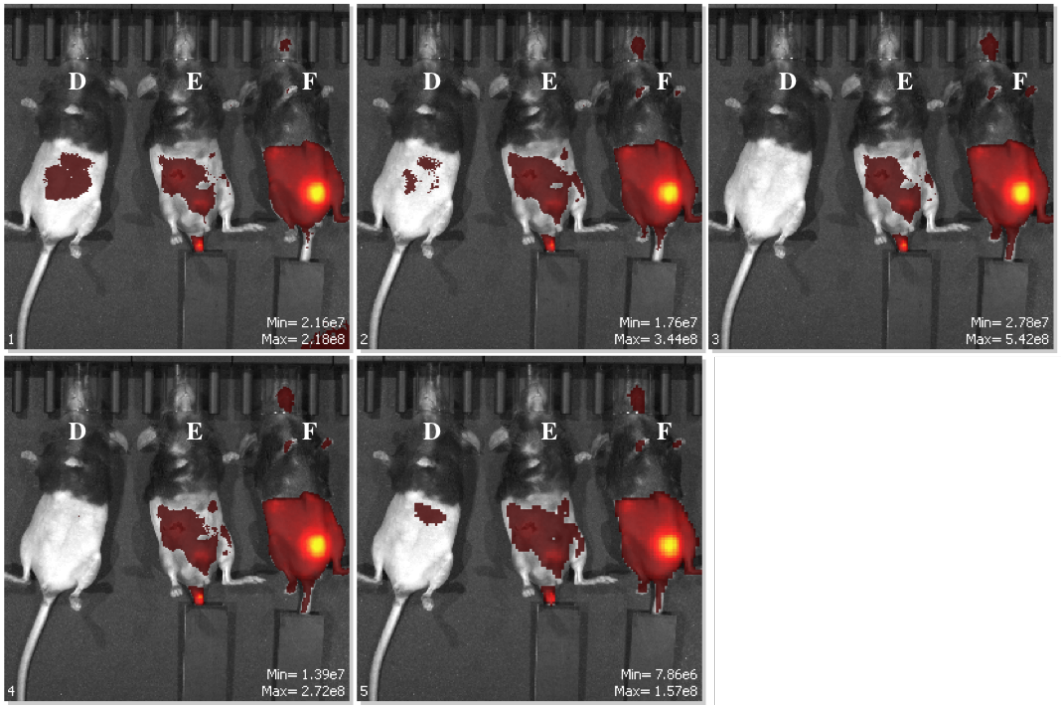

Figure 3.2.1.2 In vivo NIRF images of mice injected with ProSense 750 EX including controls. All pictures present the same animals, each image has different intensity of color scale; D - AOM/DSS treated, no probe injected (auto-fluorescence control); E - AOM/DSS treated, ProSense750 EX IP injected; F - untreated, ProSense 750 EX injected (non-specific signal control).

Ex vivo imaging of colons collected from mice injected with ProSense 750 EX provided pictures indicating that the probe binds to the tumors, giving strong and distinguishable signal (Fig. 3.2.1.3). The picture to the left shows colon tissue cut open, containing tumors imaged by the brighter, yellowish spots. Other picture from Figure 3.2.1.3 represents the same colon tissue (b) compared with intact colon without tumors (a) and fragment containing tumors inside (c). Again, stronger signal can be seen at the spots of tumors occurrence.

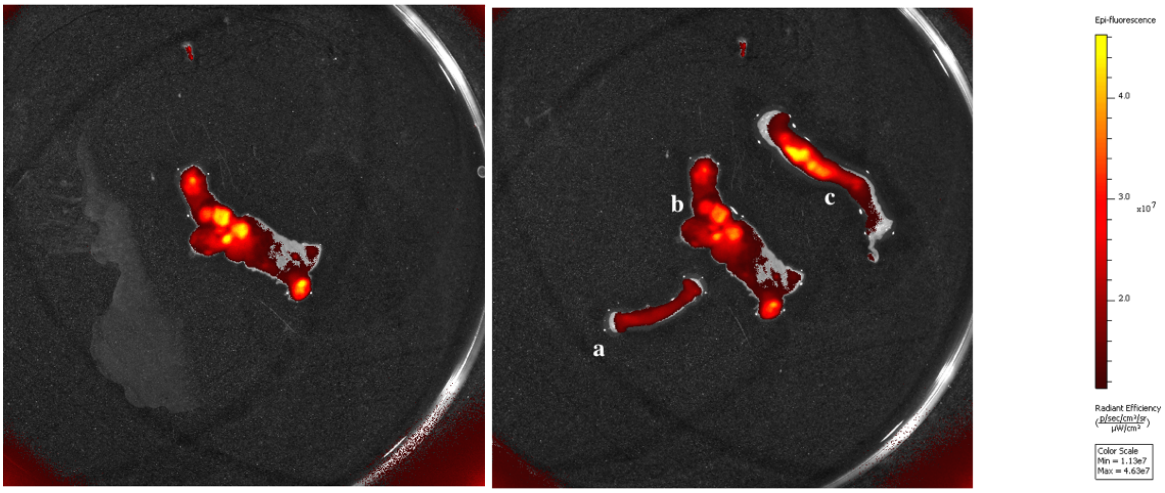

Figure 3.2.1.3 *Ex vivo* NIRF images of resected colon tissue. Colons resected from AOM/DSS treated, ProSense 750 injected mouse. Picture to the left presents colon tissue, cut open, with tumors. Picture to the right images comparison of different tissue types; a - non-tumor colon tissue; b - colon tissue, cut open, with tumors; c – colon tissue with tumors.

3.3.2 **IntegriSense 680**

2D pictures were obtained by *in vivo* NIFR imaging of mice injected with active probe IntegriSense 680. Pictures of different color intensity indicated non-specificity of provided signal. All imaged mice were injected with the probe, including one non-treated with AOM/DSS (II), and all of them provided strong background signal with some strong spot signal, identified as non tumor-originating one.

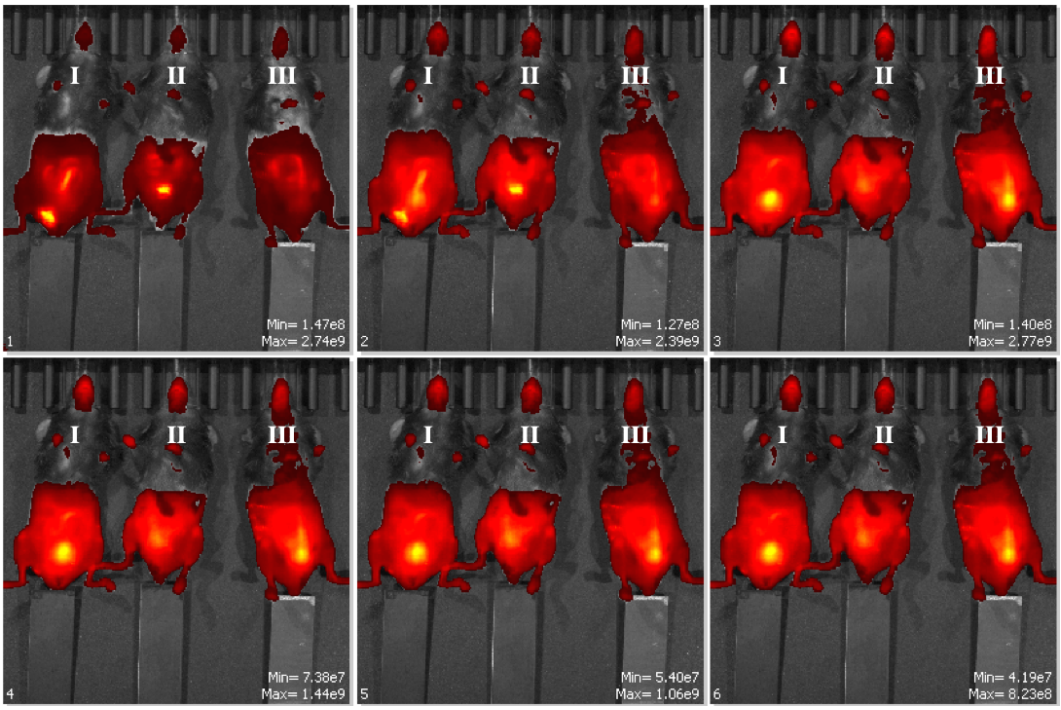

Figure 3.2.2.1 *In vivo* NIRF images of mice injected with IntegriSense 680 including controls. All pictures present the same animals, each image has different intensity of color scale; I - AOM/DSS treated, IntegriSense 680 injected; II - untreated, IntegriSense 680 injected; III - AOM/DSS treated, IntegriSense 680 injected.

Ex vivo imaging of colon tissues confirmed results from *in vivo* imaging with IntegriSense 680. Pictures presented in Figure 3.2.2.2, image colon tissue containing tumors (I) and intestines fragment (cecum) containing faces (II), showing no specific signal given by the tumor and strength of the one originating from feces.

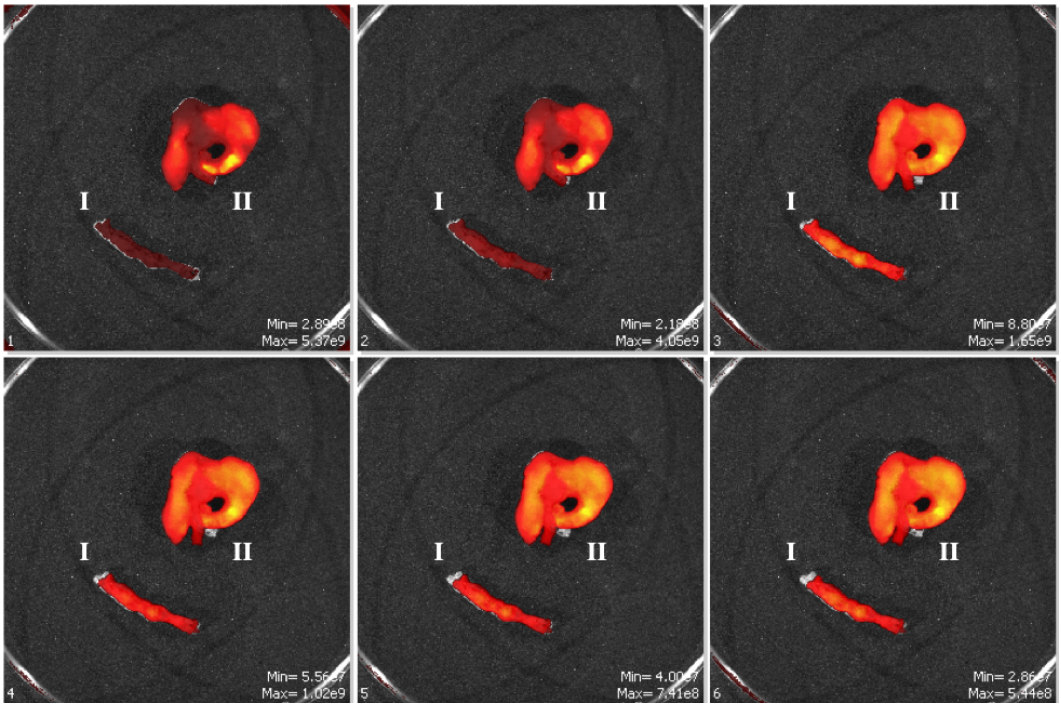

Figure 3.2.2.2 *Ex vivo* imaging of colon tissue. Colons resected from AOM/DSS treated mouse, injected with IntegriSense 680. All pictures present the same specimens; each image has different intensity of color scale; I – colon tissue with tumor; II - intestines fragment filled with feces.

3.4 AOM/DSS Insulin Receptor knockout mice model of CAC

3.4.1 Genotyping

By agarose gel electrophoresis of DNA fragments, purified form mice and amplified in a PCR reaction, genotyping of obtained breeding offspring was determined. Evaluation of loxP sites, flanking 4th exon of INSR gene, was driven by the presence of the band representing size of about 145 bp (Fig. 3.3.1.1; a-k, t-w). Non-flanked, wild types had replicate of 105 bp size (Fig. 3.3.1.1; l-s). As only single band was detected for each of the genotyped mice (Fig. 3.3.1.1), it can be concluded all of them were homozygotes in terms of loxP sites presence.

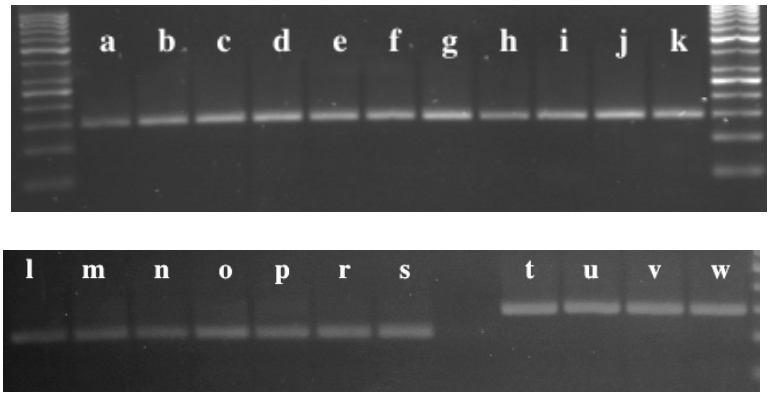

Figure 3.3.1.1 Agarose Electrophoresis of DNA amplified with primers set enabling differentiation between mice with loxP sites flanking INSR 4th exon and wild types not bearing loxP sites. Each letter indicates respective mouse.

Expression of tissue specific Cre recombinase transgene was identified by presence of band indicating 195 bp sized replicate. Either band was present - confirming recombinase expression. Alternatiely, it was absent, meaning there is no recombinase activity in the intestines. As seen in the Figure 3.3.1.2, some of the mice were bearing recombinase gen (a, b, d, e, f, j, l, o, r, s, t, u, w) while others were WT (c, g, h, i, k, m, n, p, v). Analysis of two PCR set-ups enabled detection of which mice were INSR-KO. Those samples, having one band of 145bp size in the set-up detecting loxP sites and having 195 bp band in Cre-recombinase set-up, were considered as INSR-KO: a, b, d, e, f, j, t, u, w.

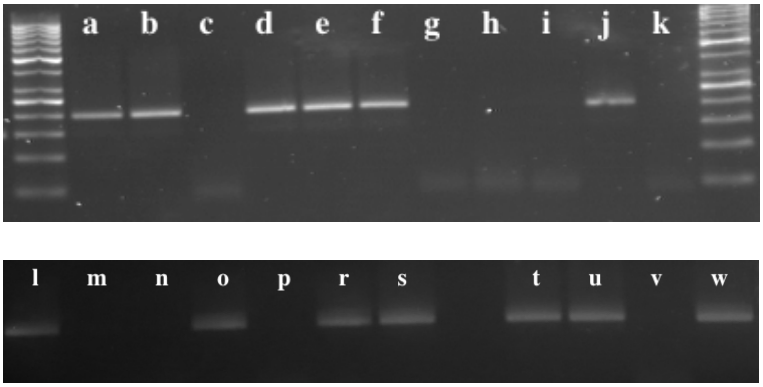

Figure 3.3.1.2 Agarose Electrophoresis of DNA amplified with primers set indicating presence of tissue specific villin – Cre recombinase. Each letter indicates respective mouse.

3.5 Organoids

Utilized method for colon crypts isolation yielded with high density of crypts in appropriate condition for initiation of 3D, Matrigel embedded organoids culture (Fig. 3.4.1 A). At the beginning (Figure 3.4.1 B), domes consisted of whole or fragmented single crypts, strongly resembling physiological morphology form. Isolated and 3D matrix embedded single cells and colon crypts survived the procedure and developed functional budding colonoids developing in time.

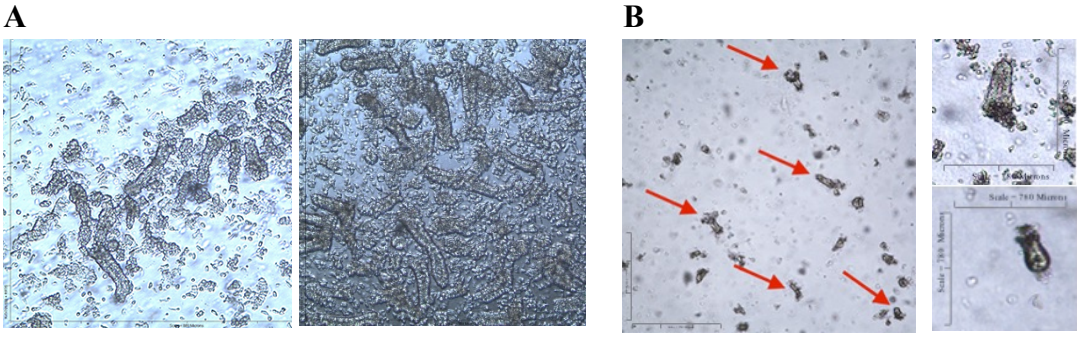

Figure 3.4.1 Colon crypts isolation and embedded cultures set up. (A) Pictures presenting step of crypts isolation where their presence is determined and further best fractions, consisting of biggest number of crypts are chosen and pulled together. (B) Isolated crypts embedded in Matrigel domes, 6 hours after setting up the culture. Red arrows in the picture to the left indicate single colon crypts. Two smaller pictures to the right illustrate fragments of separate single crypts.

Daily monitoring of the culture enabled capturing of single organoids growing over time (Fig. 3.4.2). Different shapes and growth patterns were detected in the culture of organoids (Fig. 3.4.3). Domes consisted of many cell debris and dead cells also surrounding viable crypts or developed organoids (blue arrows in Fig. 3.4.2 C: day 7; D: day 7). Nevertheless, majority of the observed organoids were growing and developing until the end of the culture.

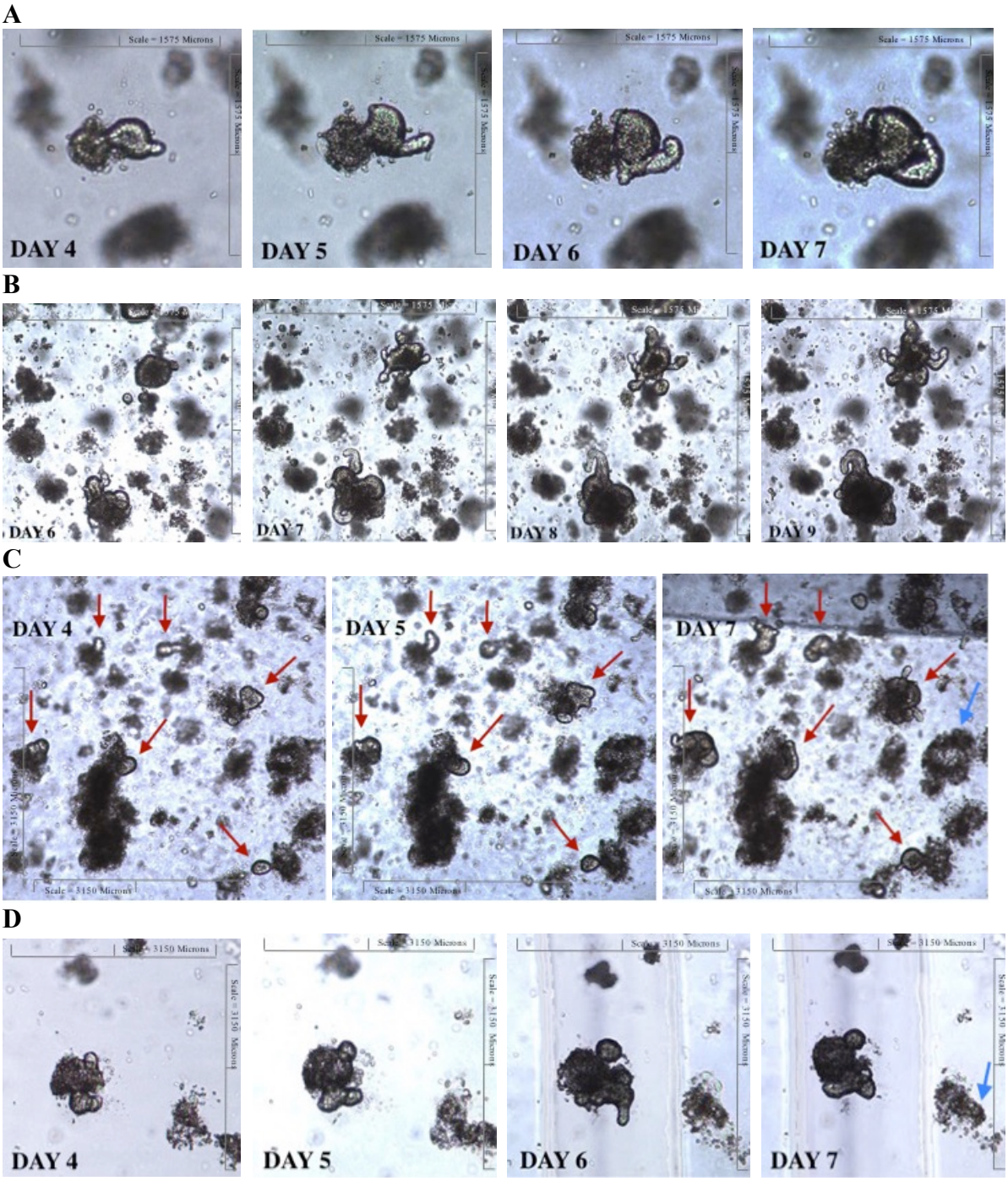

Figure 3.4.2 Colon crypts development *in vitro*. Pictures of colonoids. Respective sequences (A-D) present organoids development in the course of culturing, including particular days. (A) Single organoid development monitored over time. (B) Fragment of colon culture monitored over time. Pictures image two distinguishable, developing organoids, background is filled with death cells and debris. (C) Red arrows indicate viable, developing organoids over the time, blue arrow indicates death cells and cell debris. (D) Example of single organoid development over time. Blue arrow in the last picture (DAY 7) presents example of dead cells clump.

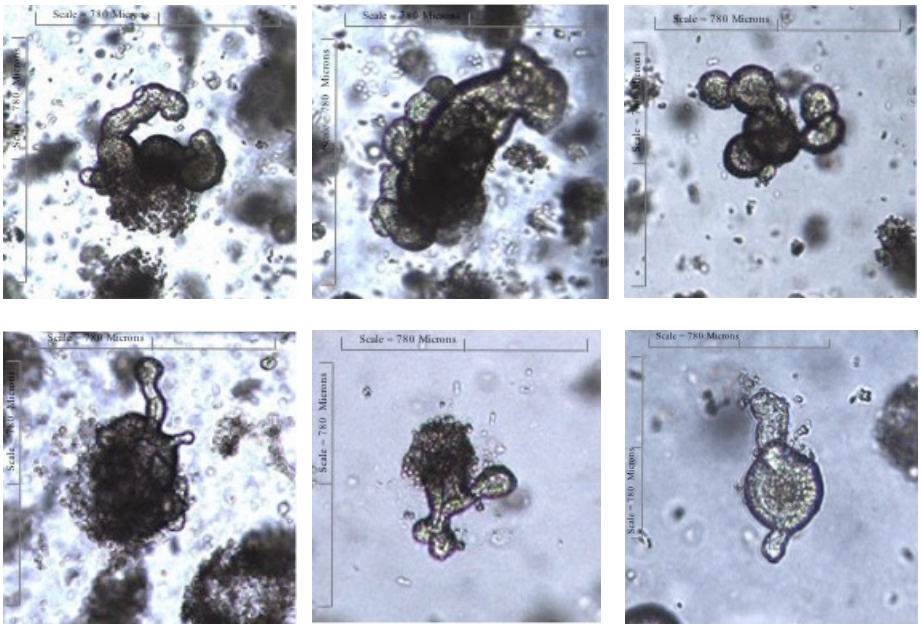

Figure 3.4.3 Individual organoids. Images of independent colonoids, at different stages of the culture development.

4 Discussion and Conclusions

4.1 Mice models of CAC and INSR knockout mice

As stated in the introduction part, chemically induced model of colitis associated colon cancer is a well-known and established method. The present study confirmed usefulness of this model. Mice treated with injected AOM and provided with 3% DSS in drinking water in three 7-days cycles developed tumors enabling modeling of colitis associated colon cancer. As seen in the weights curve (Fig. 3.1.1), mice followed expected weight loss following each DSS cycle. Additionally, during DSS treatment, rectal bleeding and diarrhea, which become chronic, confirming severity of the inflammation. Toxic action of this colitogen is known to be manifested by weight loss and bloody diarrhea in the first phase of AOM/DSS therapy (Tanaka et al., 2003b). As it is known, inflammation influences tumor promotion, initiation and progression also in non-colitis associated cancers (Quante and Wang, 2008). By this DSS application in our utilized model becomes important, not only because of colitis-associated character of research but also for efficiency of tumor development. DSS alone has been used to mimic ulcerative colitis associated cancer. However, this method is much less efficient than the combination of AOM/DSS in terms of the speed of tumor development (Okayasu et al., 2002). The combination of AOM and DSS makes the process of colorectal carcinogenesis faster and more efficient with regard to tumor number (Meira et al., 2008). Moreover, it includes the initial phase of acute inflammation, which can be detected by the morphology of colon epithelium and typical inflammatory cells presence at the site of colitis (Tanaka et al., 2003b). Results obtained from endoscopy and tumor counts supported a successful chemically induced tumor generation. Tumor counts indicated majority of the malignancies present in distal part of the colon and fulfilled the expectation of higher number of smaller tumors occurrence. As stated in the background (1.2.1) AOM/DSS mouse model strongly resembles pathogenesis of human CRC with the exception of metastasis. Pictures of dissected colons and tumor counts obtained in this project confirm this statement, as detected tumors were frequently located in distal part. On the whole, obtained results confirm generation of a model that can serve as a useful tool in colitis associated colon cancer research.

The genotyping of animals bred with intention to obtain insulin knockout model of CAC, confirm that Cre-lox recombination technique enables obtaining desired knockout mice models. Backcrossing of obtained offspring leads to selection of homozygotic in terms of loxP sites and Cre recombinase bearing individuals. As stated in the background section (1.2.2) there are alternative methods for introduction of loss-of-function genetic modification. The more recent and modern one is previously mentioned CRISPR-Cas9 technique. Comparing to the method used in this study, CRISPR enables obtaining of animals with desired genome in shorter time and with less expenses. There is no need of backcrossing and selection of obtained offspring. Gene-modified mice can be obtained in 4 weeks while Cre-lox KO requires around 6 months to be optimized. Furthermore, it is more assessable and easier to modify when compared with other site specific-methods employing nucleases as this one uses RNA as a guide recognizing

targeted sequence. However in CRISP-Cas 9 method, there is the risk of nonspecific binding yielding undesired mutation (Yang et al., 2014). As loss of function models is very useful and broadly used, this knockout brings an advantageous way of colitis associated cancer mechanism and basis exploration.

4.2 IVIS

Results obtained from IVIS experiment indicate that activable probe ProSense 750 EX binds to colon tumors specifically. This is proven by the pictures representing colon specimens (Fig. 3.2.1.3), where tumors give much stronger and intensive fluorescent signals when compared to non-tumor tissue. Nonetheless, images obtained from *in vivo* imaging do not follow that pattern and show strong signal that has been determined as non-specific one, originating from other than tumors source. That conclusion was driven by the fact that same strong signal was detected in treated and untreated mice (Fig. 3.2.1.1 mouse B- treated; Fig. 3.2.1.2 mouse F- untreated). Additionally, the location of the strong signal was recognized as not compatible with expected location of colon tumors, as these usually occur more distally, towards rectum. Strong signal was suspected to originate from the bladder, making this probe higly unspecific in our case.

Imaging with the use of IntegriSense 680 probe gave the result of high background signal and unspecific strong signal, this time having source in feces present in mice intestines (Fig. 3.2.2.1). Undoubtedly, pictures of dissected colons clarified unspecific signal originated from feces and no distinguishable signal was observed at the tumors site (Fig. 3.2.2.2). Depending on probe type, there will be possible different complications related to specificity of the signal. For instance, active probes emit fluorescence wherever they are located. The only requirement for them to emit signal is to absorb light of appropriate wavelength. Thereby they can give signal even if they are not bound to the molecule they are specific to and suppose to detect. In other words it is enough for them to be present in the system. By contrast, activable probes in fact emit fluorescence in response to enzyme activity, and therefore will not shine unless have reached the target. Despite that, there is a possibility that the probe, after being activated can diffuse in some other area, away from the target. Those problems can severely affect detected signal and increase level of non-specificity.

Another complication connected with specificity of the signal is the half-life of the probe within the organism. Any probe injected to the animal, after reaching its destination is subjected to general circulation in the organism. Depending on how and if substance is decomposed, it has to be cleared from the body. Consequently, this raises the issue of potential signal accumulation in organs as bladder, kidneys or liver, which are involved in body clearage. In this case proper timing of the imaging becomes crucial. There are many available fluorescent probes that require various times after administration and before being imaged (Hilderbrand and Weissleder, 2010). Finding appropriate time for imaging, in order to capture specific signal before molecule is subjected to excretion but after time needed for it to reach the target, seem to be very challenging. As mentioned before, strong signal obtained by *in vivo* imaging of ProSense 750 EX, even though unspecific (suspected to originate from the bladder), proved it is possible to be captured from the inside of the living animal. This empha-

sises the posibility to optimise imaging timing and capturing the probes signal, while still bound to the tumors, not yet cumulated elsewhere.

The issue of unspecific signal rises additionally from mentioned before light absorbing and autofluorescent tissues and compounds. While the problem with black fur can be solved with thorough hair removal and depilation, chlorophyll originating from diet seem to be much more influential than expected. Even though mice were fed with chow-free diet, it can be seen that feces give quite strong signal. As this appeared only in the case of IntegriSense and was not an issue for ProSense injected mice, it can be stated that reason for this unspecific signal is the spectral window used for imaging IntegriSense probe.

In conclusion, although ProSense 750 EX didn't bring desired *in vivo* imaging results, probe seems to bind specifically to the colon tumors. It can be concluded that the fluorescent signal emitted by the tumor tissue, nonetheless distinguishable and stronger than the background signal, was not strong enough to be detected *in vivo*, IntegriSense 680 did not bind to the colonic tumors either *in vivo* or *ex vivo*. It follows that it would exclude this probe as potential use in research on CRC.

4.3 Colon Organoids Cultures

The main objective of this part of the project was to establish and optimize a protocol to isolate colon crypts and set up 3D Matrigel-based culture. The utilized setup enabled culturing of isolated crypts for 9 days. As mentioned in the introduction intestinal epithelium, in particular colon crypts are very problematic to grow *ex vivo* and require providing special treatment and conditions in order to remain livable and developing. The commercial media is designed to grow organoids from the small intestine. Therefore, we supplemented it with Wnt-3 ligand – found to be a critical factor controlling stem cells self-renewal, since colonic crypts lack the Paneth cells producing this factor (Reya and Clevers, 2005). As it was suggested, it is insufficiently produced by the colonoids (Sato et al., 2011), thus its addition was a key factor for successful sustaining of the culture and organoids growth. Pictures included in results part (Section 3.4, Fig. 3.4.1) prove that colon crypts survived the isolation, and created variously shaped organoids, developing and changing in time. It can be seen that organoids created differently shaped multicellular structures (Fig. 3.4.3). Owing to the use of Matrigel - generating 3D environment, supplemented with appropriate additives, growth of isolated crypts eventually forming organoides was possible. As a result, one gains enormous amount of possibilities, resolving boundaries connected with current limitations of *in vitro* colon crypts growth.

5 Future work

In vivo imaging is a promising and powerful tool for tracking development of CAC in mice models. However, optimization and the right choice of fluorescent probes is a crucial and challenging step. Therefore, attention should be placed on development of specific, valuable probes for IBD and CRC detection. It could be done by means of personalized fluorescent probes discovery based on screening of ideal biological markers for those conditions. Fluorescence imaging is especially valuable and worth improving due to potential for facilitating not only research on CRC and IBD but also diagnosis of those in patients when introduced into the clinic. The remarkable alternative for imaging CRC in animal models is the use of bioluminescence. By generation of transgenic animals one can easily and efficiently image tumors at any stage of development. This can be done by genetic engineering of tumor cells, transferred into the animal via grafting. The gene coding luciferase enzyme is introduced into the genome of tumor cells. Prior to imaging, the animal is injected with enzyme's substrate – luciferin and light is emitted as a consequence of the catalyzed oxidation. Described bioluminescence is very advantageous in terms of no background signal emission. That can be possible as there is no need for shedding any light on the animal and the point of measurement is to capture the light emitted only by examined point of interest. Those traits of bioluminescence minimize above-mentioned issues of fluorescence. Although this method requires additional modifications within the animal, it yields much more specific and precise results than fluorescence imaging (Fernández Y, June 22, 2015)

As previously stated, organoids bring up many possibilities for specific and precise research of colon. Firstly, optimization of this cell culture type should be done. As suggested by Sato and colleagues (2011), supplementary step during crypts isolation, where additional mild digestion treatment of the crypts before culturing is proposed, yields higher plating efficiency. That would improve culture set up and facilitate analysis by reduction of dead cells and debris. Moreover, as suggested by authors, it would minimize blocking of living cells by dead ones.

The results provided in this project indicate different types of colonospheres being generated. Obviously, investigating mechanisms of crypt formation would be very useful in terms of stem cells research, as they are known to be highly involved in cancer. The next step will be to perform mechanistic studies such as signaling and therapeutic studies on cultured colon organoids.

As mentioned before, it has already been proven that growing of both physiological and pathological colon epithelium is possible. Undoubtedly both types of colonoids could be examined in many ways bringing enormous opportunities of exploration. Combining this type of crypts culturing with powerful laboratory animals disease modeling also gives wide variety of opportunities. This is possible as animals with different genetic background can be introduced as the donors, enabling specific and precise approach.

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