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THE PORCINE ANIMAL MODEL GOES THROUGH THE 3RS: DEVELOPMENT OF IN VITRO AND EX VIVO SYSTEM TO STUDY VASCULAR BIOLOGY

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Contents

ABSTRACT

Since the publication of the book of Russell and Burch in 1959, scientific research has never stopped improving itself with regard to the important issue of animal experimentation.

The European Directive 2010/63/EU "On the protection of animals used for scientific purposes" focuses mainly on the animal welfare, fixing the Russell and Burch's 3Rs principles as the foundations of the document. In particular, the legislator clearly states the responsibility of the scientific community to improve the number of alternative methods to animal experimentation.

The swine is considered a species of relevant interest for translational research and medicine due to its biological similarities with humans. The surgical community has, in fact, recognized the swine as an excellent model replicating the human cardiovascular system.

There have been several wild-type and transgenic porcine models which were produced for biomedicine and translational research. Among these, the cardiovascular ones are the most represented.

The continuous involvement of the porcine animal model in the biomedical research, as the continuous advances achieved using swine in translational medicine, support the need for alternative methods to animal experimentation involving pigs.

The main purpose of the present work was to develop and characterize novel porcine alternative methods for cardiovascular translational biology/medicine.

The work was mainly based on two different models: the first consisted in an *ex vivo* culture of porcine aortic cylinders and the second consisted in an *in vitro* culture of porcine aortic derived progenitor cells. Both the models were properly characterized and results indicated that they could be useful to the study of vascular biology. Nevertheless, both the models aim to reduce the use of experimental animals and to refine animal based-trials.

In conclusion, the present research aims to be a small, but significant, contribution to the important and necessary field of study of alternative methods to animal experimentation.

Key words: *Porcine Animal Model, Alternative Methods, 3Rs, Mesenchymal Stromal Cells, Perivascular Cells, Vascular Biology*

OVERVIEW ON TRANSLATIONAL RESEARCH AND PORCINE MODELS FOR BIOMEDICAL PURPOSES

Alternatives to Animal Testing: from the 3Rs of Russell & Burch to the European Directive 2010/63/EU

Alternatives to animal testing are an important and essential tool for scientists from all over the world that need to go toward a continuous process of development and implementation.

According to its standard definition "animal testing" is intended as "the use of animals in experiments and development projects usually to determine toxicity, dosing and efficacy of test drugs before proceeding to human clinical trials".

In a wider meaning, the phrase "alternatives to animal testing" is used by the scientific community to indicate the possibility to develop alternative methods to animal experimentation.

It is clear that animal testing cannot be reduced to the mere use of animal in studying the pharmacokinetics and pharmacodynamics of drugs. There is a huge number of scientific papers in the literature describing the use of laboratory animals to study physiology, pathology and pathogenesis of human and animal diseases (McGonigle & Ruggeri, 2014).

Russell and Burch were the first who drafted the basic principles concerning the use of animals in scientific experiments in their book "The Principles of Humane Experimental Technique". The authors described the amazing growth of biological sciences after the Darwin Revolution and the vast number of animals used for experimentation in human and veterinary medicine and in basic sciences. Overall, the authors underlined within the aim of their work that "the humanest possible treatment of experimental animals (…) is actually a prerequisite for successful animal experiments" (Russell and Burch, 1959), unifying, for the first time, animals rights and researchers main goal.

The main inheritance that Russell and Burch's work left to the scientific community was, indeed, the so-called 3Rs: Replacement, Reduction and Refinement.

The term Replacement refers to the possibility of using any scientific method that does not employ animals to reach the same results that were reached using conscious living vertebrates. If an alternative exists, researchers are not ethically allowed to use animal in their experiments.
The term Reduction refers to a meticulous and continuous activity leading to the reduction of experimental animals numbers (whenever they cannot be Replaced), in order to achieve the same results with different means. The reduction is described as "the one most obviously, immediately, and universally advantageous of all modes of progress in terms of efficiency".
Lastly, the term Refinement refers to the detailed study of animal experimentation in order to choose the right procedures and the right species for the experiments, and in order to minimize animals stress and pain. This final stage, in fact, aspires to look for the continuous enhancement of the animal wellness (Russell & Burch, 1959).

Since the publication of the book of Russell and Burch in 1959 the research in the 3Rs field has never stopped improving itself with regard to the important issue on animal experimentation Over the past 40 years the Russell and Burch's 3Rs have commonly been accepted as ethical guidelines and as milestones for all the animal-based studies, in many countries all over the world (Forni, 2007; Singh, 2012).

In this regard, the commitment taken by the scientists attending the 2010 "First Basel Conference" is of major importance. The same commitment was, then, formalized into the Basel Declaration and signed by many other scholars all over the world through its web page (http://www.basel-declaration.org). This document states that modern science still needs to use animals in order to study physiology, pathology and pathogenesis of diseases and to improve veterinary approaches. Nonetheless, some fundamental principles should be observed. Surely, the 3Rs prove to be the kind of ethical principles the scientific community is looking for, whose main goal is the respect for all the animals used for experimental purposes, trying to avoid them pain

and useless suffering. Moreover, the declaration encourages collaboration among scientists, in order to avoid repeating futile animal-based experiments, as well as the education of the people involved in animal experiments. Finally, with the declaration it is stated that necessary research involving animals have to be allowed in the future and it is asked to encourage the public discussion of sensitive issues concerning research involving animal (Basel Declaration, 2010).

Right now, animal models are considered the major source of biological and physiological information, a precious system for drug discovery and, consequently, of utmost importance as a translational tool in most of the major human diseases (Wendler & Wehling, 2010).

Despite the progress in animal experimentation procedures, the ethical acceptance of animal models for the study of human diseases and, eventually, their treatment is still into focus. Animal testing, in fact, has always been one of the main actors in the international scientific arena. Concerning it, one of the key questions is the validation of the model in term of transferability to the human species (Dothel et al., 2013).

The translation of theoretical knowledge and experimental breakthroughs into clinical practice of medicine is also known as Translational Medicine. Obviously, scientific discoveries prove to be complete when they result into an application: usually, a medical discovery follows the path, from the “benchside to the bedside” resulting in the so called Translational Research (Keramaris et al., 2008). Animal testing places itself exactly in the middle of the process of the Translational Research applied to the study of human biology.

Evidently, the best model to study human biology (physiology, pathology, treatment of diseases) would be the human subject, but it is clear that this option can not be considered ethical. To overcome this problem, animals have been routinely used as models to characterize pathogenesis of diseases and to discover new drugs, entailing several advantages such as the homogeneity of the population and the wider possibilities to study toxicity of treatments, pathology and physiology. The

evaluation of mechanism of action of existing drugs and the discovery of new drugs have been widely carried out using animal models. However, even if the animal is a complex and functional model, effective treatments on animals could be less or not effective or, sometimes, even harmful on humans (Hooijmans & Ritskes-Hoitinga, 2013; McGonigle & Ruggeri, 2014). The main challenges in Translational Research consist in the biological differences among species, the poor methodological quality of animal experiments, the differences in designing the experiments that involve animals, as well as the deficient reporting of the details concerning the animal experimentation (Hooijmans & Ritskes-Hoitinga, 2013).

Scientists are always committing to the continuous challenge faced by the translational research involving animal experiments. In 2002 the LANCET journal reported the article "Systematic reviews of animal experiments" written by Sandercock and Roberts. This paper strongly recommended the systematic review of the literature concerning well performed animal experiments (high quality methodology) as the base of any new clinical trial (Sandercock & Roberts, 2002). It is quite clear that the high quality methodology (Russell and Burck's Refinement) is essential to improve the outcome of a translational research.

The urgent need for guidelines in animal experiments was also underlined in the Kilkenny and colleagues paper. After a survey on a consistent number of scientific papers concerning animal testing, the authors reported a lack of some of the major information about the experiments that involve animals and recommend an accurate and transparent description of the methods (Kilkenny et al., 2009). In their following articles, they also defined the Animal Research Reporting In Vivo Experiments (ARRIVE) guidelines as a tool for animal-based experimentation (Kilkenny and colleagues, 2010 and 2012; Hooijmans & Ritskes-Hoitinga, 2013; Dothel et al., 2013), available on the web (https://www.nc3rs.org.uk/arrive-guidelines) and actually required by many relevant biological journals for publications of animal experiments.

Despite the enormous efforts that many scientists did in the last decades, the current debate focuses mainly on the question: are preclinical studies in animals still ethically acceptable (Dothel et al., 2013)?

Unfortunately, the present question requires more than a simple answer. As the topic is of clear interest, not only for the scientific community but also for those who do not belong to it, a specific legislation about animal protection is necessary.

In Europe, the previous Directive of the European Commission in the field regarding the animal protection was released on 24 November 1986 (Directive 86/609/EEC). Its adoption, nonetheless, varied according the different Member States. Some of them, for instance, implemented it with measures to ensure higher level of protection for the animal used for experimental purposes. However, these disparities within Member States have been considered a possible barrier for the exchange of products and substances that involve animal-based trials.
Moreover, since 1986, new scientific data have been collected, new technological approaches have been used and new evidences updated the knowledge about animal welfare and the capacity of animals to express pain, suffering and distress.
Considering these and other relevant factors, the European Union released the DIRECTIVE 2010/63/EU on September 22nd, 2010 "On the protection of animals used for scientific purposes" that substitutes Directive 86/609/EEC.
The new Directive focuses mainly on the animal welfare fixing the 3Rs principles as the foundations of the document.
The theme of animal welfare is analysed in all its form, from the design of the experiment and the consequent training of the staff, to the housing of the animal and the control of its pain, and again from the animal breeding to the animal human sacrifice.
Moreover, although the document affirm that the use of live animals in experimental trials continues to be necessary to protect human and animal health, the European Commission clearly states the responsibility of the scientific community to improve the number of methods that allow the replacement of live animals in experimental procedures.

Finally, “this Directive represents an important step towards achieving the final goal of full replacement of procedures on live animals for scientific and educational purposes as soon as it is scientifically possible to do so” (DIRECTIVE 2010/63/EU). The Italian law adopted the Directive with the “Decreto Legislativo n. 26, March 4th, 2014”, which contains more restrictions regarding Italian scientific research.

The Porcine Model in Translational Research

A simple PubMed research using the keywords "porcine/swine/pig animal models" reveals that in the last three decades there has been an exponential growth of papers in which the swine was considered a species of relevant interest for translational research and medicine (and sometimes the species of choice – Prather et al., 2013).
Papers' topics are the most different: the pig has been and still is used as model for studies in fields of embryology, physiology, pathology of human disease, nutrition, toxicology and agriculture. Nevertheless, the Directive 2010/63/EU also regulates the use of the pig, in animal-based experiments, as a not-primate animal species (Directive 2010/63/EU).

As previously described in the present manuscript, an animal model, in order to be considered a valid tool for translational research, should replicate at most the human condition (Suzuki et al 2011). However, concerning this point, it is important to keep in mind that "All models – the biological ones, too – are wrong: the practical question is how wrong do they have to be to not be useful " (Box, 1987; Prather et al., 2013). Considering that the Directive 2010/63/EU clearly states that "the use of live animals in experimental trials continues to be necessary to protect human and animal health" (Directive 2010/63/EU), it is still important that scientists continue to make efforts in their researches in order to find the least worst model to represent human conditions without using live animals for biomedical purposes.

The mouse is, nowadays, an irreplaceable model and absolutely necessary for advances in biomedicine. However, as human and mouse are quite different under many biological aspects, the generation of large animal models able to properly mime human diseases is needed (Fan & Lai, 2013; Prather et al., 2013).
It is already known since many years that pig is more similar to the human species than other not-primate animals (Swindle et al., 2012). Moreover, it seems that the use of porcine models could present fewer ethical issues and societal concern than the use of primates and pets (i.e dogs) in biomedical trials, probably due to their use as livestock and food producing animals (Fan & Lai, 2013; Prather et al., 2013).

The biological similarities with humans are expressed at the genomic (Archibald et al., 2010; Bendixen et al., 2010), proteomic (de Almeida et al., 2012), anatomic and physiologic levels (Niemann & Kues, 2003; Suzuki et al., 2011; Swindle et al., 2012). In addition, farm pigs and minipigs have a longer lifespan than the smaller laboratory animals (Fan & Lai, 2013). In particular, due to their smaller size the minipig strains are the appropriate choice when scientists need a more manageable animal/species for their research. Moreover, minipigs seem to be a better choice, when compared to farm pigs, because they are more mature, although with the same body weight, and because their tissues are more resilient to experimental procedures (Swindle et al., 2012; Lelovas et al., 2014).
Nonetheless, disadvantages in the use of the pig as animal model, opposed to mouse and rat, lie in higher costs as well as their more complex handling, late maturity and long gestational period (Elmadhun et al., 2013).

Looking it from a translational point of view, the porcine species has been recognized, indeed, as an excellent model for the study of cardiovascular diseases (Zaragoza et al., 2011; Elmadhun et al., 2013) and it has also led scientists to improve their knowledge about atherosclerosis and thrombosis pathogenesis and treatment (Vilahur et al., 2011; Hamamdzic & Wilensky, 2013), myocardial ischemia (Suzuki et al., 2011) and restenosis (Gessaroli et al., 2012; de Prado et al., 2013).

Human eye pathologies, such as Leber Congenital Amaurosis and other inherited retinal degenerations, could also benefit from the use of pig model. Pig retina, in fact, displays a macular region similar to the human one, confirming in terms of morphology and function, its being a good model in this field of research (Fan & Lai, 2013; Prather et al., 2013; Gün & Kues, 2014). An Italian group funded by Telethon grants, is currently working on these topics and developing several adeno-associated virus-based *in vivo* transgene delivery pig models in order to use the so-called gene therapy as a way to treat retinal diseases (Karali et al., 2011; Mussolino et al., 2011; Manfredi et al., 2013).

There is also plenty of literature in which the porcine model is employed to study lung diseases. Some major topics, for instance, focused on lung development and transplantation, ischemia-reperfusion injury, nitrous oxide and hyperoxia effect, pulmonary hypertension, endothelin and its receptors, asthma and other diseases (Rogers et al., 2008 a).

Moreover, it is important to remember that a close similarity between human and porcine species was also described for the gastrointestinal trait (Weih et al., 2013). Pig has proved to be a good model in pediatric gastroenterology (Sangild et al., 2013), especially in the research field on the short bowel syndrome (SBS) (Sangild et al., 2014).

Furthermore, it is worth citing the enormous contribution of genetically-modified pig animal models to biomedicine (Whyte & Prather, 2011); the advanced reproductive technologies available for this species (Sachs and Galli, 2009), the knowledge about its genome (Archibald et al., 2010) and the new tools the genetic engineering offers to scientists (Petersen et al., 2015). All these features make the pig model a powerful tool for the translational research (Fan & Lai, 2013).

The recent advances in regenerative medicine and tissue regeneration led to the use of the pig animal model in this field of study too. The porcine animal model has been employed, especially, in cardiovascular (see i.e. Simioniuc et al., 2011) and orthopedic (see i.e. Sun et al., 2013) regenerative medical trials.

In 2003, the NIH, taking into account all these considerations, established the National Swine Resource and Research Center (NSRRC) “to develop the infrastructure to ensure that biomedical investigators across a variety of disciplines have access to critically needed swine models of human health and disease” (http://www.nsrrc.missouri.edu). The NSRRC is probably the clearer example of the significance of the pig as an animal model for human diseases, given that it also serves as a central resource for reagents, information and training related to the use of swine models in biomedical research (Prather et al., 2013).

The following chapter presents a review of the literature concerning pig animal models. In particular, the following section will focus on the most important application of the porcine model in biomedicine, that is its relevance in cardiovascular diseases research field. It will also analysed useful porcine models for biomedical purposes obtained with genetic engineering and porcine models application in regenerative medicine.

Wild-type porcine models for cardiovascular research

Cardiovascular diseases are the first causes of death (3 out of 10 deaths) all over the world (data related to 2000-2012 published in the WHO website http://www.who.int/en/). Consequently, nowadays, one of the major fields of study of the scientific research is the cardiovascular physio-pathology. In order to perform successful research, it is necessary to have a model that accurately approximates to the human cardiovascular morphology and function (Lelovas et al., 2014). The surgical community has hence recognized the swine as an excellent model replicating the human cardiovascular system (Prather et al., 2013).

The features that make the swine an attractive model for cardiovascular studies can be found in the high similarities with humans in coronary anatomy and circulation, in the physiology of the heart, in hemodynamic and metabolic values, in pharmacokinetics and platelet function, coagulation and fibrinolysis. In addition, pigs tolerate invasive cardiac interventions, as well (Elmadhun et al., 2013; Fan & Lai, 2013; Gün & Kues, 2014; Lelovas et al., 2014).

Atherosclerosis and myocardial infarction can be experimentally induced in swines through diets enriched with the nutrients known for their property to elevate the risk for cardiovascular disorders in humans (Prather et al., 2013). Furthermore, it was demonstrated how some pigs develop spontaneous atherosclerotic lesions and may succumb to a sudden death when under stress (Vilahur et al., 2010).

The following chapters will briefly summarized the most important wild type porcine models of cardiovascular diseases in order to examine them from the laboratory animal science point of view.

Porcine models for Atherotrombotic Disease

Atherosclerosis is a cardiovascular disease that could affect all vessels. It is considered an intima (the inner layer of the vessels) related disease and it is caused by hyperlipidemia and lipid oxidation. Fat deposits constitute the so called “intimal plaque” whose formation occurs due to the deposit of small cholesterol crystals in the intima and in the media layers of vessels. Then, the fibrous tissue and the plaque grow resulting into the vessels’ lumen obstruction. Clot formation and thrombosis

are the last stages of an atherosclerotic lesion. In particular, the possible rupture of the plaques could lead to thrombosis (atherothrombotic event) that is the most important cause of acute coronary syndrome and ischemic heart disease (Vilhaur et al., 2011; Zaragoza et al., 2011; Hamamdzic et al., 2013; Rafieian-Kopaei et al., 2014).

Porcine models of atherosclerotic lesion have been properly reviewed by Zaragoza and colleagues (2011) and by Hamamdzic and colleagues (2013). The following part of this work briefly describes them in order to underline the most important ones.

In order to induce the atherothrombotic events in swine, it is possible to feed animals with an hypercholesterolemic diet that increases their cholesterol levels resembling the human pathological ones. Thanks to the porcine model, it is also possible to combine this approach with intravascular interventions (balloon angioplasty and stenting) in order to induce an adaptive response in pigs that resembles the human phenotype (Zaragoza et al., 2011). Examples of the use of the latter approach can be found in the Busnelli and colleagues work, were they investigated hypercholesterolemia from the inflammatory point of view (Busnelli et al., 2013).
The hypercholesterolemic pig model just presented is usually coupled with the diabetic pig model (Hamamdzic et al., 2013) firstly described by Gerrity and colleagues (2001). Combining the two diets, hypercholesterolemic and hyperglycemic, results demonstrate that lesions closely resemble the human phenotype (Hamamdzic et al., 2013).
A second porcine model of atherosclerosis is the so-called Rapacz-FH pig (Prescott et al., 1991). This model exhibits spontaneous atherosclerosis due to high blood levels of low density lipoprotein (LDL). This spontaneous phenotype is caused by a mutation in the LDLR gene and the consequent developments of atherosclerotic lesions and pathological phenotypes are highly similar to the human one (Prescott et al., 1991; Hamamdzic et al., 2013). Recently, the Rapacz-FH pig model has also been used to study the gene expression profile of valvular interstitial cells (Porras et al., 2014).

The last and noteworthy porcine model of atherosclerosis is the so-called Ossabaw Pig. The Ossabaw Pig develops metabolic syndrome due to a non-insulin spontaneous diabetes associated with an atherogenic diet. Coronary atherosclerosis was observed in this model by Hamamdzic and colleagues in 2013 (Hamamdzic et al., 2013). Recently, the Ossabaw pig has been used to investigate the cardiac angiogenesis in a chronical ischemic myocardium (Elmadhun et al., 2014).

Porcine models for Myocardial Ischemia

One of the main outcome of the rupture of an atherosclerotic plaque is the acute coronary syndrome and the consequent myocardial ischemia, due to ischemic heart disease (Hamamdzic et al., 2013). Coronary Artery Disease (CAD) is the first cause of death all over the world. This is due to two major consequences that occur in about the 50% of cases: acute Myocardial Infarction (MI) or sudden cardiac death (Iwasaki, 2014). MI is considered the main consequence of myocardial ischemia which, finally, leads to cell death (myocardial injury) (Whyte et al., 2014).

The main issue to overcome, when using animals to model myocardial infarction pathogenesis in order to develop useful treatments, is the recreation of the so-called "vulnerable atherosclerotic plaque". The pig seems to be the best species to generate the plaque instability (Suzuki et al., 2011; Zaragoza et al., 2011). Several porcine models of plaque rupture method are thoroughly detailed in the Zaragoza and colleagues review (Zaragoza et al., 2011).

In addition to these previous models, induction of myocardial ischemia in pigs can be achieved through other approaches, among which the most commons are the surgical occlusion of the coronary artery and the induced supraventricular tachycardia (Suzuki et al., 2011; Zaragoza et al., 2011).

Moreover, cardiac catheterization and coronary interventions are similar in pigs and humans. So, in order to induce heart failure and to develop procedures and treatments for cardiovascular diseases, the possibility to work on the pig, instead of other animals, with a catheter-based closed-chest technique proves notable advantages. (Suzuki et al., 2011).

Porcine models for Restenosis

In order to treat Coronary Artery Disease (CAD), which follows the accumulation of atherosclerotic plaque within the lumen of carotid arteries, the most common procedure is the use of stents. The main problem of the coronary stent is that it causes restenosis (Perkins et al., 2010).

Generally speaking, restenosis is a process that occurs in about 10-15% of patients treated with coronary, carotid and peripheral arteries revascularization procedures (Forte et al., 2014). The main pathogenic event occurring in restenosis is the development of the neointimal hyperplasia. It appears that neointimal hyperplasia could be due to the overgrowth of vascular smooth muscle cells and/or multipotent vascular cells migrating from the media layer of the vessels to the damaged intimal one (Giordano & Romano, 2011; Tang et al., 2012). An inflammatory component was also recognized in the development of neointimal hyperplasia (Giordano & Romano, 2011).

So far, the porcine coronary model has been one of the most useful for the evaluation of coronary stents, as its development of the neointimal injury pathology is seen as the closest to the human one. Furthermore, it is important to remember that, due to the adult pig size, stents and all instrumentations used for humans are equally suitable for pigs (Vilahur et al., 2010; de Prado et al., 2013).

Recent studies in which pigs were used as restenosis models aimed mainly to develop methods or devices to be employed in cardiovascular surgery.

To this end, Cui and colleagues (2014) and Li and colleagues (2014) described the employment of particular stents coated with antibodies in a porcine model of restenosis. The aim of the studies was to develop new devices in order to overcome the de-endothelialization problem and prevent restenosis.

Pérez de Prado and colleagues (2014), instead, assessed paclitaxel-eluting balloons efficacy in a preclinical porcine model achieving the reduction of the restenosis in treated animals compared to the control.

Furthermore, Gessaroli and colleagues (2012) used the porcine model to assess a modified ePTFE stretch graft in order to prevent restenosis in vein-graft anastomosis.

Transgenic porcine models for human diseases

Although the first transgenic pig was generated about thirty years ago for agricultural purposes, in the biomedical research field the swine is considered a species of great interest insomuch as, nowadays, biomedicine is the main application of genetically modified pigs (Sachs & Galli, 2009; Whyte & Prather, 2011; Prather et al., 2013; Gün & Kues, 2014).

In particular, it is recognized that transgenic pigs are better animal models than other non-primate species in fields concerning neurological, cardiovascular, and diabetic disorders (Fan & Lai, 2013).

Recent advances in reproductive biotechnologies applied to porcine species, genetic engineering techniques and molecular biology tools allowed scientists to generate transgenic pigs in a faster way than few years ago (Galli et al., 2010; Gün & Kues, 2014).

In the last 30 years, several transgenesis techniques have been developed, ameliorated and described (Bacci, 2007; Sachs & Galli, 2009). Among these, it is worth remembering the principal ones such as the pronuclear microinjection (Gordon et al., 1980), the Sperm Mediated Gene Transfer (Lavitrano et al., 2002), the viral-mediated transgenesis (Whitelaw et al., 2004) and the Somatic Cell Nuclear Transfer, based on the animal cloning technique (Galli et al., 2010).

The main problem with the porcine species in terms of transgenesis is the lack of Embryonic Stem Cell (ESC) lines which otherwise would provide a simpler way to generate transgenic animals (Park & Telugu, 2013). An important tool aiming to ameliorate the transgenesis efficiency in pigs could be the emerging induced Pluripotent Stem (iPS) cell lines, already described for the porcine species (Roberts et al., 2009; West & Stice, 2011; Fan & Lai, 2013).

Improvements in molecular biology knowledge and techniques led scientists to develop innovative technologies based on transposons, recombinases and nucleases (Gün & Kues, 2014). Nucleases constitute the most powerful tools emerged in the last few years. Among them Zinc-Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs) and RNA-guided Engineered Nucleases (RGENs) are the most used systems (Kim & Kim, 2014; Petersen et al., 2015). Moreover,

recently, the CRISPR/Cas9 nuclease system has been employed in order to generate genetically modified pigs (Hai et al., 2014; Whitworth et al., 2014).

In the last two decades, transgenic pigs were generated and perfected as some of them resulted in great animal models for human diseases. Three different reviews concerning transgenic pigs in biomedicine were recently edited by Fan and Lai (2013), Prather and colleagues (2013), and Gün and Kues (2014). The following sections will briefly expose the data presented in these reviews updated with the most recent and noteworthy scientific publications. This comprehensive revision will thus examine transgenic pig models for cardiovascular, nervous system and ophthalmological diseases, diabetes mellitus, cystic fibrosis, xenotransplantation and cancer.

Cardiovascular Diseases

The present manuscript has already described the similarities in terms of cardiovascular morphology and function between the human and the porcine species. In the last decade, transgenic pig models for cardiovascular diseases were generated, in order to allow scientists to deeply investigate the etiology and to accelerate the discovery of new therapies for some of these multifactorial and complex pathologies (Zaragoza et al., 2011; Agarwala et al., 2013; Prather et al., 2013).

Polyunsaturated fatty acid n-3/n-6 ratio is of great importance in the cardiovascular field. The two models described below could help in investigating the effect on the cardiovascular system of an altered n-3/n-6 ratio (Prather et al., 2013). A spinach Δ12 Fatty Acid Desaturase (FAD2) expressing transgenic pig was generated by Saeki and colleagues in 2004. Naturally, mammalians do not express desaturase, whose function is to produce the essential polyunsaturated fatty acid Linoleic Acid and α-Linoleic Acid (Saeki et al. 2004). Moreover, a humanized *Chaernobitis elegans fat1* expressing transgenic pig was generated by Lai and colleagues; *fat1* encodes a n-3 desaturase and this lead to the alteration of polyunsaturated fatty acid n-3/n-6 in the transgenic pig compared to the wild-type (Lai et al., 2006).

An important gene involved in cardiovascular biology is the endothelial Nitric Oxide Synthase (eNOS), an enzyme expressed by endothelial cells that generates Nitric

Oxide (NO). Nitric Oxide is significant as a regulator of vascular morphology and function (Hao et al., 2006; Whyte et al, 2010). Catalase is another enzyme expressed by endothelial cells that contributes to vascular tone and to the development of cardiovascular diseases metabolizing H_2O_2, another important regulator of vascular physiology (Whyte et al., 2011).

Transgenic pigs expressing eNOS (Hao et al., 2006; Whyte et al, 2010) and catalase (Whyte et al., 2011) were generated by two different research groups. Both the animal models could provide important information about the contribution of NO and H_2O_2 to cardiovascular diseases and could improve the knowledge about their physiological function.

Peroxisome Proliferator-Activated Receptor-γ (PPARγ) is involved in cardiovascular diseases and is considered a valid target for therapeutic interventions due to its role in fatty acid and adiponectin uptake in adipocytes. Moreover, the activation of PPARγ results in an increase of insulin sensitivity and glucose uptake and in an anti-inflammatory effect (Fan & Lai, 2013; Prather et al., 2013; Gün & Kues, 2014). Hence, with the generation of the PPARγ-KO pig, Yang and colleagues provided to scientists an important tool to get new important information on the role of PPARγ in cardiovascular biology (Yang et al., 2011).

An important independent risk factor for coronary heart disease is the hypertriglyceridemia and Apolipoprotein (Apo)CIII, which is strongly correlated with plasma triglyceride levels. Due to these important considerations, Wei and colleagues produced an ApoCIII transgenic pig model that showed important similarities with the human pathological phenotype and could be of great importance for studies on correlation of hyperlipidemia and atherosclerotic diseases (Wei et al., 2012).

Another interesting molecule involved in cardiovascular biology is CD39 (ectonucleoside triphosphate diphosphohydrolase-1). Its involvement in cardioprotection was demonstrated in a transgenic murine model of myocardial ischemia/reperfusion. In 2012, Wheeler and colleagues generated a CD39 transgenic pig in order to investigate the translational potential of the murine model. The results obtained after a myocardial ischemia/reperfusion injury confirmed, as showed in the

murine model, that CD39 is involved in cardioprotection in the ischemic heart disease (Wheeler et al., 2012).

Recently, Al-Mashhadi and colleagues generated a transgenic pig modified for the expression of a mutated form of the convertase subtilisin/kexin type 9 (PCSK9) gene that causes, in human, hypercholesterolemia and atherosclerosis. The model displayed the same human pathological phenotype and could provide an important tool to study atherosclerosis (Al-Mashhadi et al., 2013).

Nervous System Diseases

Transgenic pigs have been generated for Alzheimer's Disease (AD), Spinal Muscolar Atrophy (SMA), Huntington's Disease (HD) and Amyotrophic Lateral Sclerosis (ALS).

Alzheimer's Disease pathogenesis is not entirely known, but it seems that Amyloid Precursor Protein (APP) gene is a key factor in the development of this neurodegenerative disorder, which also leads to the loss of neurons and cognitive function and, at a later stage, to death. Genetically modified pigs have been generated through the insertion of the Swedish mutation of the human APP gene. Unfortunately, no symptoms related to AD and no symptomatic β peptide accumulation have been yet observed in the system; the potential manifestation of Alzheimer related symptom may take more time to develop in the present model (Kragh et al., 2009; Søndergaard et al., 2012).

Spinal Muscular Atrophy is a neurodegenerative disease whose main cause is recognized in a mutation or a deletion of the Survival Motor Neuron (SMN)-1 gene, which is characterized by loss of motor neurons and skeletal muscle atrophy (Fan and Lai, 2013). To this end, a transgenic pig lacking just one allele of the SMN1 gene was recently generated. Obviously, the requirement of the double KO of the SMN1 gene is essential in order to simulate the human phenotype of the disease (Lorson et al., 2011).

Huntington's Disease pathogenesis is related to the expansion of the CAG trinucleotide in the Huntingtin (HTT) gene. The disease results in a progressive loss of brain neurons that leads to neurological disorders related to movement, cognitive ability and dementia.

Two models of this disease were obtained by Yang and colleagues (2010) and Baxa and colleagues (2013). In particular, in the first the insertion of mutant HTT resulted in the birth of piglets with a low expression of the mutant gene, but with clear evidence of apoptosis in brain neurons.

Amyotrophic Lateral Sclerosis is a fatal neurodegenerative disease and can be clinically distinguished into two forms: sporadic and familiar. The 20% of the familiar forms seems to be linked to a mutation of the Cu/Zn Superoxide Dismutase (SOD)-1 gene. A transgenic pig model carrying and expressing the human SOD1 mutated gene was recently generated and characterized by Chieppa and colleagues (2014).

Ophthalmological Disease

Retinitis Pigmentosa are a group of inherited retinal diseases that cause loss of peripheral and, sometimes, central vision, mainly due to the loss of photoreceptors (rods for peripheral and cones for central vision) (Fan & Lai, 2013; Prather et al., 2013; Gün & Kues, 2014).

There were two main transgenic pig models generated for retinitis pigmentosa. The first one was generated by Petters and colleagues introducing a mutation in the Rhodopsin (RHO) gene that causes, in the pig retina, rods loss, cone photoreceptors degeneration and consequently the loss of their function (Petters et al., 1997). The second model was generated by Fernandez de Castro and colleagues, introducing a different mutation in the RHO gene and this resulted in a pathological phenotype completely similar to the human one, confirmed from the electroretinographes (Ross et al., 2012; Fernandez de Castro et al., 2014).

Diabetes Mellitus

Among metabolic disorders, Diabetes Mellitus is the most represented and it is mainly characterized by hyperglycemia. In particular, Type I, II (both insulin-dependent) and III (insulin-independend) of Diabetes Mellitus were described and characterized by Wolf and colleagues (2014).

Recently, Renner and colleagues generated a porcine model of permanent neonatal diabetes introducing a mutation in the pig genome at the insulin (INS) gene level. The genetically modified newborn pigs showed hyperglycemia and a decreased body weight and β cells mass. Due to these features, this model could be of great interest in clinical trials (Renner et al., 2013).

Transgenic pigs for Glucagon-like peptide (GLP)-1 were also generated. It is important to remember that GLP1 protein is involved in the secretion of insulin. These pigs showed a continuous reduction in glucose tolerance and in the number and mass of β cells with increasing age (Renner et al., 2010)

Moreover, pigs were genetically modified in order to investigate the mutated Hepatocyte Nuclear Factor (HNF)-1α recognized as the cause of Diabetes Type III by Umeyama and colleagues (2009). These pigs developed diabetes due to a poor insulin secretion.

Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive genetic disease caused, mainly, by the mutation in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene encoding for a ion channel protein whose function is to mediate hydration. The pig was proved to be a good animal model on which studying human lung diseases (Rogers et al., 2008 a & b).

There are two main transgenic pig models which were developed for this pathology; the first was generated through the deletion of the porcine CFRT gene by Rogers and colleagues (2008 b) while the second was generated through the mutation of the CFRT gene by Ostedgaard and colleagues (2011). Both models, amazingly, resemble the human CF phenotype in terms of lung, liver, pancreas, bladder, bile duct, intestinal and vas deferens pathologies.

However, the greatest results were achieved with the first model, which allowed scientists to deeply investigate CF pathogenesis. In particular, it was demonstrated that inflammation follows bacterial infection in CF patients lungs (Stoltz et al. 2010), that Insulin-like Growth Factor (IGF)-1 is correlated with CF (Rogan et al. 2010) and that mucociliary transport is a primary defect in CF patients lungs (Hoegger et al., 2014).

Xenotransplantation

The continuous request for organs, due to the high number of people that need transplants, led scientists to look for other ways to get organs donors. Xenotransplantation might be identified as a possible solution to the problem: getting organs from other species clearly allows an increase in their number in order to supply to this pressing request. Pig has been described as the most suitable species for xenotransplantation because of its size, breeding and the possibilities to modify its genome in terms of addition and deletion of genes (Sachs and Galli, 2009; Prather et al., 2013). In fact, the first of all transgenic pigs produced for biomedical purposes was generated for xenotransplantational studies by Cozzi and colleagues (1997).

There are, however, two main problems related to organ xenotransplantation from pigs: the humoral rejection (Le Bas-Bernardet et al., 2011) and the presence of porcine endogenous retroviruses – PERV (Mattiuzzo et al., 2012).

Rejection of xenotransplants from porcine to primates is basically due to the so-called process of hyperacute rejection. Hyperacute rejection is mainly driven by pre-existing human (and primate) antibodies able to recognize α-gal epitopes catalyzed by the α-1,3 galactosyl transferase (GGTA)-1 gene, which lead to rejection in a matter of minutes (Sachs and Galli, 2010; Prather et al., 2013; Gün & Kues, 2014).

The production of the first Gal-KO pig in the 2003 by Phelps and colleagues (Phelps et al., 2003) increased the survival of the transplanted organs (2-6 months), but the modulation of other complement regulatory proteins (CRPs) is still necessary (Le Bas-Bernardet et al., 2011). Recently, other attempts to produce transgenic pigs and use their organs in xenotransplantational studies using the Gal-KO background were made and some of them showed encouraging results (Bongoni et al., 2014). However, it is clear that a lot of work has to be made in order to overcome the immunologic and zoonotic issues that xenograft presents.

Cancer

The word “cancer” defines a series of more than 100 pathologies that share the uncontrolled cell growth as their principal feature. Different types, with different pathogenesis, of human cancer are known and well described (Gün & Kues, 2014).

So far, there have been generated some porcine cancer models even if the pig has not yet played a critical role in experimental oncology (Flisikowska et al., 2013).

For instance, pigs were genetically modified for the Brest Cancer Associated Gene (BRCA) 1 in order to provide a model for the study of the breast and ovarian cancer. Unfortunately, the usefulness of this model still needs to be demonstrated, as all the piglets died within 18 days after birth (Luo et al., 2011).

Another transgenic pig was generated by Leuchs and colleagues in order to mime the Li-Fraumeni syndrome. This pig was generated with a conditionally activatable oncogenic mutant of the p53 oncogene that plays an important role in human cancer. Leuchs and colleagues observed that the model resembled very closely the human phenotype (Leuchs et al., 2012).

However, the most impressive transgenic pig model generated so far is the one in which the Adenomatous Polyposis Coli (APC) gene was targeted. A mutation in the porcine APC gene (orthologous to a severe mutation in the human APC gene) closely resembles the Familial Adenomatous Polyposis (FAP) human phenotype. The founder animal revealed polyps in colon-rectum similar to human adenomas (Flisikowska et al., 2012). Other analysis are still in progress within the F1 generation (Flisikowska et al., 2013).

Involvement of the porcine model in regenerative medicine

The NIH defines regenerative medicine "*a broad field that includes tissue engineering but also incorporates research on self-healing – where the body uses its own systems, sometimes with help foreign biological material to recreate cells and rebuild tissues and organs*" (http://www.nibib.nih.gov/).
Basically, "*Regenerative medicine replaces or regenerates human cells, tissue or organs to restore or establish normal function*" (Mason & Dunnill, 2008).
This replacement could be achieved through several approaches among which stem cell transplantation, gene therapy, tissue engineering, soluble molecules delivery, cell reprogramming (Greenwood et al, 2006).
Thus, it is clear that one of the milestones in regenerative medicine is definitely the cell and, in particular, the stem cell. Depending on their commitment, stem cells can be classified into totipotent, pluripotent, multipotent or progenitor/tissue specific cells. There are many clinical trials already approved for therapy on humans and many other that are currently object of studies (Mason & Dunnill, 2008; Sanchez et al., 2012). In particular, many encouraging results have been achieved in the field of cardiovascular regenerative medicine, insomuch as cell-based phase III clinical trials have been started (Behfar et al., 2014). Great advances have also been achieved within the field of bone engineering through mesenchymal stromal cell therapy (Grayson et al., 2015).

The use of the porcine animal model was described in several biomedical works concerning regenerative medicine in both cardiovascular and orthopedical fields.

Porcine animal model and cardiovascular regeneration

Regenerative medicine is a promising way to overcome cardiovascular diseases even though the many issues it still presents nowadays (cell graft composition, cell selection, cell differentiation) and that need to be overcome (Dai & Foley, 2014).

The pig has been used as a model for cardiovascular regeneration in order to overtake some of these issues.

A pig model of balloon induced-myocardial infarction was used by Grøgaard and colleagues in order to evaluate how the injected cell homed to the infarcted tissue. Autologous progenitor cells were intravenous or intracoronary injected. Results showed a greater number of infused cells in infarcted myocardium after intracoronary than intravenous injection (Grøgaard et al., 2007).

Instead, data obtained by Halkos and colleagues, who again used a pig model of myocardial infarction, suggest that intravenous injection of MSCs stimulate reperfusion of the ischemic myocardium resulting in the improvement of the cardiac function (Halkos et al., 2008). Similar results were also achieved by Krause and colleagues who obtained, with a similar method, a reduction of the infarct size and, again, an improvement of the cardiac function (Krause et al., 2007). Moreover, data obtained from Sato and colleagues suggested a neovascularization process induced by the autologous MSCs intracoronary injection in the porcine infarcted heart (Sato et al., 2011).

Simionuc and colleagues also tested the effect of the injection of placental derived-MSCs pre-treated with a hyaluronan mixed ester of butyric and retinoic acid on infarcted porcine hearts. Infarct size were reduced as well as fibrous tissue, and vascularization was enhanced after the injection of pre-treated cells compared to the injection of untreated cells (Simioniuc et al., 2011).

In order to assess whether delivery efficiency based on the site of injection was different, van der Spoleen and colleagues injected intracoronary or trans-endocardium bone marrow MSCs in a porcine model of myocardial infarction. No differences were observed by the authors in the delivery efficiency between these two approaches (van der Spoleen et al., 2012).

Using a transgenic line of human induced Pluripotent Stem Cells (hiPSCs), modified for the expression of the sodium iodide symporter (NIS), Templin and colleagues demonstrated, for the first time in a porcine model of myocardial infarction, that hiPSCs are able to differentiate to endothelial cells contributing to the neovascularization of the infarcted heart (Templin et al., 2012).

Furthermore, by using the same model Wright and colleagues demonstrated that the delivery of alginate encapsulated MSCs, modified for the expression of a Glucagon-like peptide (GLP)-1, after myocardial infarction, improves left ventricular function,

reduces the infarct size and increases the number of vessels, probably due to a paracrine supply of GLP-1 and other stem cell factors (Wright et al., 2012).
In addition, recent studies on the porcine model allowed to assess the use of three-dimensional systems for stem cell therapy of the infarcted heart (Emmert et al., 2013).

Porcine animal model and bone regeneration

By employing animal model adult bone marrow- and adipose tissue-derived mesenchymal stromal cells (MSCs) were used in clinical trials for bone regeneration (Grayson et al., 2015).

The porcine model was used in order to assess bone regeneration in mandibular distraction. It was shown that autologous bone marrow MSCs delivery in a Gelfoam scaffold to the distraction site improves the osteogenic process in pig mandible (Sun et al., 2013). The pig model was also previously used to demonstrated that stem cells isolated from deciduous teeth are able to engraft and repair a critical lesion at the mandibular level (Zheng et al., 2009).
The porcine model helped studying cartilage defect repair, as well. In their recent study, Wang and colleagues modified bone marrow MSCs for the expression of Bone Morphogenetic Protein (BMP)-2 and Transforming Growth Factor (TGF)β3 through an adenoviral vector. Cells were cultured with demineralized bone matrix (DBN) and the resulting engineered scaffolds were used for surgery in pigs on which a knee defect had been surgically generated. Results obtained demonstrate that the DBN engineered with MSCs modified for the expression of BMP2 and TGFβ3 elicit the cartilage repair *in vivo*. Authors state that this could be a possible clinical treatment for cartilage damage (Wang et al., 2014).

Relevant porcine models developed at the BCM laboratory

The BCM laboratory (Biotecnologie Cellulari e Molecolari) was founded at the University of Bologna by Professors Forni and Bacci about 20 years ago. The present work was written while attending this facility. Since its foundation, the BCM laboratory has been dealing with *in vivo*, *ex vivo* and *in vitro* porcine models, developed for translational purposes.

Initially, transgenic pig models through SMGT were generated within the field of xenotransplantation (Lavitrano et al., 1999; Lavitrano et al., 2002; Webster et al., 2005; Manzini et al., 2006; Smolenski et al., 2007; Vargiolu et al., 2010).

Moreover, *in vivo* and *in vitro* porcine models were used, by the research group, to investigate the physiology of reproduction (Bacci et al., 1996; Bernardini et al., 2003; Forni et al., 2003; Bernardini et al., 2004; Zannoni et al., 2006; Ribeiro et al., 2007 a, Ribeiro et al., 2007 b; Zannoni et al., 2007).

Recently, it was also studied some aspects of the porcine gastrointestinal physiology (Dall'aglio et al., 2011; Dall'aglio et al., 2013) and pig was used as a model to study the chronic ingestion of toxin contaminated food (Bernardini et al., 2014).

In vivo and *ex vivo* porcine models were developed, in the cardiovascular field, in order to study the effects of carbon monoxide (CO) in an ischemia/reperfusion injury model (Lavitrano et al., 2004) and in an endotoxic shock model (Mazzola et al., 2005; Forni et al., 2005; Zannoni et al., 2010; Zannoni et al., 2012). In the field of cardiovascular diseases, an *in vivo* model of mild hypercholesterolemia was developed in order to study vascular injury (Busnelli et al., 2009; Busnelli et al., 2013).

In vitro cultures of porcine Aortic Endothelial Cells (pAEC) were developed by the research group, to study the vascular response to different shock (Bernardini et al., 2005; Bernardini et al. 2010; Bernardini et al., 2012).

AIM OF THE STUDY

The main purpose of the previous chapter was to thoroughly describe the pig animal model in the most of its features and applications in translational research.
In particular, it has been underlined the great similarity between the porcine and the human species within the field of cardiovascular physio-pathology. Moreover, it has been discussed the main reasons why the pig is considered an excellent model for translational medicine by the scientific community.
As clearly stated in the European DIRECTIVE 2010/63/EU on the protection of animals used for scientific purposes "it is desirable to replace the use of live animals in procedures by other methods not entailing the use of live animals, the use of live animals continues to be necessary to protect human and animal health and the environment (DIRECTIVE 2010/63/EU – [10])". At the same time, the directive states that: "The availability of alternative methods is highly dependent on the progress of the research into the development of alternatives. The Community Framework Programmes for Research and Technological Development provided increasing funding for projects which aim to replace, reduce and refine the use of animals in procedures (DIRECTIVE 2010/63/EU – [46])".
It is clear from these statements that the request of the European Union to the scientific community is to develop and characterize new alternative methods to animal experimentation.
Considering these requests and the twenty year experience of the BCM laboratory in managing pig animal models, the main purpose of this PhD thesis was to develop and characterize novel porcine alternative methods for cardiovascular translational biology/medicine.
The work has been based mainly on two different models: the first consists in an *ex vivo* culture of porcine aortic cylinders and the second consists in an *in vitro* culture of porcine aortic derived progenitor cells.

The development and the characterization of the *ex vivo* aortic cylinders method (organ culture) was carried out with the aim to provide a new tool for the study of cardiovascular physio-pathology, in particular for the study of vascular restenosis. Specific culture conditions were set up in order to resemble the physiological context

(vessel wall) and to overcome some limitations of the standard *in vitro* culture of endothelial and vascular smooth muscle cells.

The isolation and characterization of progenitor cells from porcine aorta was performed with the aim to provide a new *in vitro* system to study cardiovascular biology, from both physiological and pathological points of view, considering the importance of the pig in the regenerative medicine field. The aim of that work was to establish a newer and simpler method for isolating and culturing mesenchymal stromal-like cells from the porcine aorta. In order to employ this new method in the field cardiovascular translational research, a fine characterization of the differentiation potential of these cells was considered of particular importance.

The following two chapters will describe the two methods, respectively.
In particular, the aortic organ culture method has already been published in *ATLA – Alternatives To Laboratory Animals Journal* and in 2013 it was selected as the best article published in the journal ("Dorothy Hegarty Award 2013").
The isolation and culture method for mesenchymal stromal-like cells from porcine aorta has been published in *American Journal of Physiology (AJP) – Cell Physiology*, while their full differentiation potential characterization has been described in another article submitted to the same journal.

DEVELOPMENT OF A VESSEL ORGAN CULTURE SYSTEM TO STUDYCARDIOVASCULAR BIOLOGY

Brief overview on the research

As stated in the aim chapter, the description on the developed vessel organ culture system and the characterization of it was published in *ATLA*, in 2013 (Zaniboni et al., 2013). The following pages will report the entire article in order to give the reader an exact description of the method and of the characterization results.

The method was based on the idea to develop a model that could resemble, *in vitro*, the cardiovascular physio-pathological condition that occurs *in vivo*. This system was developed in particular for studies within the field of vascular restenosis.

As already explained an *in vitro* system cannot fully resemble the enormous complexity of a whole organism.

Within the cardiovascular research field, endothelial cells are considered the principal players in physiological and pathological conditions. Therefore, our attempt with this vessel organ culture system was to overcome the most important limitation of the primary endothelial cell culture, that is the lack of the physiological context: the vessel wall.

Clearly, our endeavor to propose a new vessel organ culture system is not the first one.
Vessel organ culture methods have been already described in the past (Gotlieb & Boden, 1984; Koo & Gotlieb, 1991; Merrick et al., 1997; Lyubimov & Gotlieb, 2004; Gelati et al., 2008; Bellacen & Lewis, 2009), although no one considered the fact that the vessel wall is made up of different cell types with obvious different metabolical and nutritional requests. The novelty of our methods consists exactly in this: we cultured cylinders of pig aorta using two different culture media, liquid for the inner layer and semi-solid for the outer layer.

The characterization of the system stability was based on molecular biology and histological techniques in order to analyze the production of molecules related to the

endothelial cell biology and to identify possible pathological cells (i.e. myofibroblasts involved in restenosis [Shy et al., 1997]).

It was then possible to demonstrate the stability of the organ culture system, thus offering the scientific community a new tool to study the cardiovascular physio-pathology, as well as new possible treatments for vascular diseases.

Lastly, in 2014, the scientific contribution of our work to the 3Rs of Russell and Burch was awarded with the "Dorothy Hegarty Award 2013" by the editorial board of *ATLA*. The Award is presented annually to the authors of the paper published the previous year in *ATLA* which is likely to make the most significant contribution to the reduction, refinement and/or replacement of animal experimentation.

In conclusion, with the present work we reached our goal. We set up a new method for the study of vascular biology also abiding by the request of the EU Directive 2010/63/EU to develop and characterize new *in vitro – ex vivo* models.

Article

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"Dorothy Hegarty Award 2013"

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Development of a Vessel Organ Culture System: Characterisation of the Method and Implications for the Reduction of Animal Experiments

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Summary — In the field of cardiovascular research, the pig is considered to be an excellent animal model of human diseases. It is well-known that primary cultures of endothelial cells (ECs) are a powerful tool for the study of vascular physiology and pathology, and, according to the principles of the Three Rs, their use results in a substantial reduction in the numbers of experimental animals required. However, a limitation of EC culture is that the cells are not in their physiological context. Here, we describe and characterise a method for the culture of porcine vessels that overcomes the limitation of EC cultures, with the advantage of reducing the number of animals used for research purposes. The organ cultures were set-up by using an aortic cylinder obtained from the arteries of control pigs sacrificed for other experimental purposes. In order to characterise the method, vascular endothelial growth factor (VEGF) secretion, matrix metalloproteinase (MMP) activation and the vessel's structural features were evaluated during organ culture. These analyses confirm that the culture of aortic cylinder lumen, in a medium specific for ECs, results in a stable system in terms of VEGF and MMP secretion. The ECs do not undergo cell division during the organ culture, which is also the case *in vivo*, if no stimulation occurs. Overall, we show that this novel system closely resembles the *in vivo* context. Importantly, porcine aortas can be collected from either veterinary surgeries or slaughterhouses, without having to sacrifice animals specifically for the purposes of this type of research.

Key words: *aorta, endothelium, organ culture, reduction, vascular disease.*

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Introduction

In the past 20 years, there has been an exponential increase in the number of scientific research studies in which pigs were used as the animal model for human pathologies. This is mainly due to the fact that the pig is biologically more similar (in terms of anatomy, physiology and genome sequence) to humans than are other non-primate animal species (1, 2). In particular, it has been demonstrated that the pig is an excellent model for research in the field of cardiovascular disease (3–7).

It is well-known that the development of several pathologies, such as atherosclerosis, hypertension and stenosis (i.e. neointimal hyperplasia; 8–10), is strongly linked to endothelial dysfunction (11). Endothelial cells (ECs) react to stress and injury with a pro-vasoconstriction, pro-coagulation and pro-inflammatory phenotype that might instigate certain vascular pathologies. Therefore, improvements to endothelial function can lead to earlier therapeutic intervention, and can contribute to the reduction of the risk of cardiovascular disease (12, 13).

In order to study the biology of the vascular endothelium, primary cultures of ECs from different species (e.g. murine, porcine, bovine and human) are commercially available, or can be derived from blood vessels. These are powerful tools that guarantee the reproducibility of the experiments, lower costs, and significantly reduce the use of experimental animals according to the principles of the Three Rs (14). However, primary cell cultures display some limitations — in particular, the cells are not in their physiological context (i.e. they are in a vessel), and they are in a proliferative state, as opposed to a non-proliferative state *in vivo* (where the average lifespan of an EC is more than one year; 15).

It has long been known that organ culture systems could overcome these limitations — vascular

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organ cultures were described nearly 20 years ago, when vessel sections from various species were maintained in culture for several weeks (16). Moreover, as with EC primary cultures, the vessel organ cultures permit a considerable reduction in the number of animals used for research, due to the fact that vessels can be obtained from the slaughterhouse or from control animals sacrificed for other experimental purposes.

However, the organ culture methods described in the literature relied on small pieces of tissue (17–19) or used thin vessel rings for angiogenesis assays (20, 21). In all these cases, a single culture medium was used, without consideration of the fact that the blood vessel wall is made up of different types of cells.

In our study, we established a new method for the culture of porcine aortic cylinders embedded in a solid agarose matrix. This procedure permits the use of different culture media — a medium for the exterior of the vessel and another one for the lumen — in order to preserve the structure and function of all the components of the blood vessel, and in particular, the endothelium.

In order to characterise this method, in addition to classical histological analysis, we assessed the expression of some molecules involved in EC biology (vascular endothelial growth factor [VEGF], and matrix metalloproteinases [MMPs]), and molecular markers of myofibroblasts, which are cells involved in the etiopathogenesis of some vascular diseases (e.g. neointimal hyperplasia; 22).

Materials and Methods

All the reagents were Gibco® products, and were purchased from Life Technologies (Carlsbad, CA, USA), unless otherwise specified.

Animals

Thoracic aortic segments were collected from 40–45kg female Large White (LW) purebred pigs, sacrificed at our institution's operating theatre for other purposes. All of the animals were part of the control group of a nutritional trial. The arteries were selected for uniform length and diameter, according to the parameters described below.

Aorta organ culture development

Thoracic aortic segments (n = 10) of 11 ± 2cm in length were collected and washed twice with a 0.9% (w/v) NaCl, 1mg/ml ampicillin sterile solution, and then transferred to a sterile 200ml tube (Falcon; Becton-Dickinson, Franklin Lakes, NJ, USA), containing Dulbecco's Phosphate Buffered Saline (DPBS; Cambrex Bioscience Inc., Wakersville, MA, USA) with 10× (10% v/v) Antibiotic–Antimycotic (Anti–Anti; Gibco; Cat. No. 15240-062). The aortic segments were transferred to the laboratory within one hour, on ice and, from that moment onward, each manipulation took place in a laminar flow cabinet.

The aortic segments were transferred to a clean sterile 200ml tube and washed twice with DPBS + 10× Anti–Anti and once with Dulbecco's Modified Eagle Medium (DMEM; Gibco; Cat. No. 41966) + 10× Anti–Anti. The superfluous tissue and aortic branches were removed (Figure 1), and cylinders (1.1 ± 0.1cm in length and 1 ± 0.1cm in diameter) were obtained by cutting the aortas transversely in correspondence with the roots of the aortic branches (as indicated in Figure 1). The aortic cylinders were washed with DMEM + 10× Anti–Anti, then the organ culture was set-up, as follows (Figure 2a):

Two millilitres of 2% w/v agarose (Sigma-Aldrich, St Louis, MO, USA) in DPBS, previously sterilised by autoclaving and maintained at 80°C in a water bath, were spread on the bottom of each well of a 6-well plate (Becton-Dickinson). After agarose polymerisation, one aortic cylinder was fixed to the bottom of each well by using 500µl of melted 2% agarose solution in DPBS. A sterile solution (7.5 ± 0.5ml) of 1% w/v low-melting agarose (Sigma-Aldrich) in DMEM + 10× Anti–Anti (which corresponds to the external medium; EM), maintained at 39°C in a water bath, was dispensed outside the aortic cylinder. The lumen cavity was filled up with internal media (IM), which varied according to the experimental conditions described in the next section.

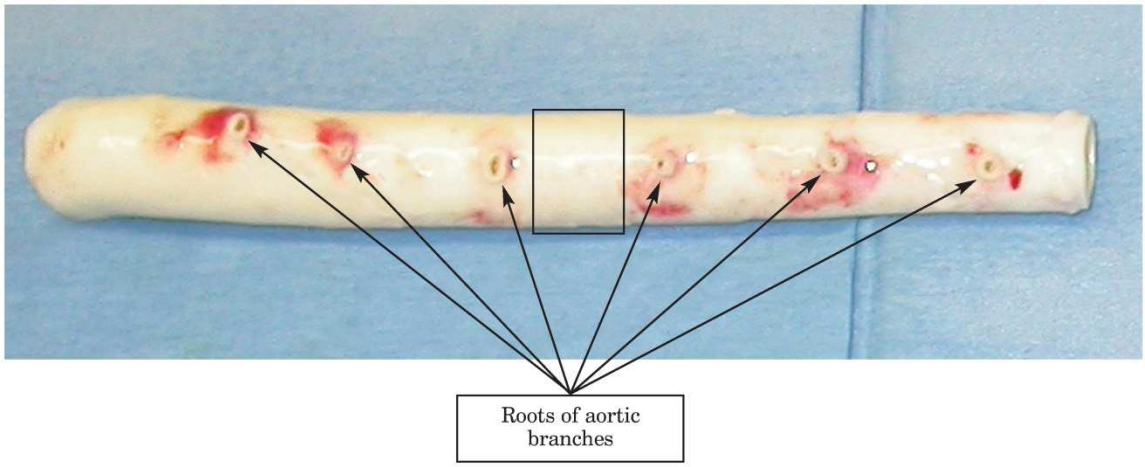
For the first 18 hours, the IM were supplemented with 10× Anti–Anti. From then on, they were supplemented with 1× Anti–Anti and changed every 2–3 days. The organ cultures were maintained in a 5% v/v CO_2 incubator, at 38.5 ± 0.5°C, for 14 days.

Characterisation of the aorta organ culture method

The effects of IM on vascular endothelial growth factor (VEGF) secretion

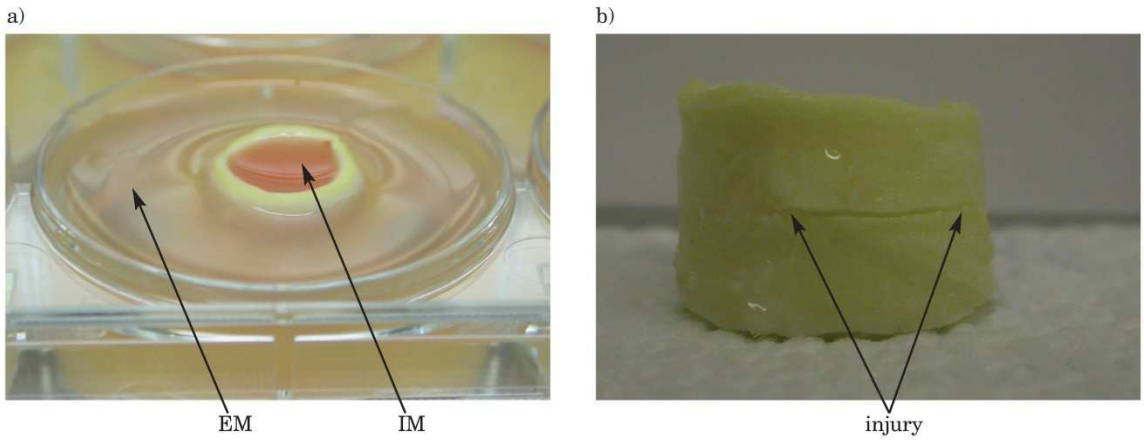
The organ cultures were performed in triplicate (n = 3) and the IM used were DMEM and human endothelial serum-free medium (heSFM; Gibco Cat. No. 11111), both supplemented with 5% v/v fetal bovine serum (FBS). At each experimental time-point (days of culture D1, D2, D5, D7 and D14), the IM were replaced. The spent media were transferred to sterile 2ml microcentrifuge tubes (Eppendorf AG, Hamburg, Germany), snap frozen in liquid nitrogen, and stored at –80°C for subsequent VEGF analysis by ELISA.

Figure 1: The porcine aortas before organ culture



The cleaned porcine aorta is shown, already free from all superfluous tissues, as described in the text. The generation of an aortic cylinder by performing transverse cuts between two roots of aortic branches is illustrated by the rectangle shown on the figure. The arrows indicate the roots of the aortic branches that have been eliminated for the generation of the aortic cylinders.

Figure 2: Organ culture of porcine aortic cylinders



The organ cultures were developed as described in the Material and Methods*, both for uninjured (control) and injured (cut) system. In a) the details of a well are shown. Note that, for culture, a semi-solid culture medium (external medium; EM) was used outside the aortic cylinder, whilst liquid culture medium (internal medium; IM) was used inside the lumen. Image b) shows a representation of an injured aortic cylinder. Note the full depth cut of 15mm in the centre of the vessel wall. The arrows in the figure show the two extremities of the injury.*

The ELISA was performed by using the Human VEGF Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA), which has already been shown to be suitable for the pig (23), with 200µl of culture medium/well. An Appliskan microplate reader (Thermo Fisher Scientific, Vantaa, Finland) was used to measure the absorbance of the samples.

The activation of MMP-2 and MMP-9 during organ culture

In order to evaluate whether the edges of a cut to the aortic cylinder could influence the organ culture system, in particular by modifying the expression and/or activation of molecules involved in cellular migration and tissue remodelling (i.e. MMPs), an injury was created by performing a full depth incision of 15mm in the centre of the vessel wall (Figure 2b).

The organ cultures were then set-up as described, for the control (uninjured aortic cylinders) and for the cut (injured) aortic cylinders. All of the cultures were performed with heSFM + 5% FBS as the internal medium (IM). The presence of active MMP-2 and MMP-9 was investigated, both in the tissues and in the IM samples (in the secreted form).

At each experimental time-point (D0, D1, D2, D5, D7 and D14), control ($n = 3$) and cut ($n = 3$) aortic cylinders were collected and a portion of tissue (9mm^2) was taken from the centre of the vessel wall. For the cut system, the tissue sample was taken exactly to correspond to the full depth cut. The tissue samples were transferred to 2ml microcentrifuge tubes (Eppendorf) and snap frozen in liquid nitrogen. At D1, D7 and D14, IM were sampled as described in the previous section.

Working on ice, 40mg of tissue were sliced, transferred to a 2ml microcentrifuge tube containing 400µl of cold sterile DPBS with protease inhibitor cocktail (Sigma-Aldrich) and homogenised with an Ultra-Turrax (IKA®-Werke GmbH & Co., Staufen, Germany) for 2 minutes at maximum speed (30-second pulses); the homogenisation step was repeated twice. The samples were then centrifuged at 10,000*g* for 10 minutes at 4°C and the supernatants were recovered.

Protein quantification was carried out with Total Protein Kit Micro Lowry (Sigma-Aldrich). For tissue and secreted MMP activation analysis, 30µg of total protein and 7.5µl of culture medium were used, respectively. Analysis of MMP-2 and MMP-9 activity through zymography was performed as previously described (24).

The effects of organ culture on the structural integrity of the vessels

An evaluation of the integrity of the vessels was performed on aortic cylinders cultured in heSFM + 5% FBS as the internal medium, at D0 and D14.

The arteries were fixed in 10% v/v formalin for 24 hours, embedded in paraffin, cut into serial sections (5µm thickness) and mounted on gelatin-coated slides for analysis by light microscopy. The sections were histologically examined, and compared to each other after staining with haematoxylin–eosin (HE) and immunoperoxidase.

The sections, mounted on gelatin-coated slides, were rehydrated through a graded ethanol series: 2 × 2 minutes in 100% ethanol, 2 minutes in 95% v/v ethanol, 2 minutes in 90% ethanol, 2 minutes in 80% ethanol, 2 minutes in 70% ethanol, 2 minutes in 50% ethanol, and 2 minutes in dH_2O. Thereafter, the sections were stained for 20 minutes in a 1% haematoxylin (Cat. No. 4302; Merck, Darmstadt, Germany) solution, differentiated in tap water and, subsequently, stained for 30 seconds in a 1% eosin (Cat. No. 1345; Merck) solution. The sections were then dehydrated through a graded ethanol series (1 minute in 50% ethanol, 1 minute in 70% ethanol, 1 minute in 80% ethanol, 1 minute in 90% ethanol, 1 minute in 95%, and 2 minutes in 100% ethanol), cleared in xylene and cover-slipped with Entellan® (Merck).

The serial sections were prepared for immunohistochemistry and, after de-waxing and blocking of the endogenous peroxidase activity (in 1% v/v H_2O_2 in DPBS for 30 minutes at room temperature), underwent antigen retrieval by incubation (twice, 5 minutes each) in citrate buffer (pH 6.0). The sections were rinsed in DPBS (three times for 10 minutes each) and, to block non-specific binding, they were incubated in a solution containing 10% v/v normal goat serum (Cat. No. CS 0922; Colorado Serum, Denver, CO, USA) and 0.5% v/v Triton X-100 (Merck) in DPBS, for 2 hours at room temperature. Thereafter, the sections were incubated in a solution containing either mouse anti-α-smooth-muscle actin (α-SMA; 1:100; clone 1A4; Cell Marque, Rocklin, CA, USA), mouse anti-smooth-muscle myosin (SMM, heavy-chain; 1:100; MAB3570; Chemicon/ Millipore, MA, USA), or mouse anti-Ki-67 (1:100; clone MIB-1; Dako, Denmark), for 24 hours at 4°C (the final concentrations of the primary antibodies were established by performing immunoperoxidase staining by using a different dilution pattern), in 1% normal goat serum and 0.5% Triton X-100, for 24 hours at 4°C. Following incubation in the primary antiserum, the sections were washed three times (10 minutes each wash) in DPBS and incubated in a solution containing goat biotinylated anti-mouse (1:200; BA-9200; Vector Laboratories, Burlingame, CA, USA), 1% normal goat serum and 0.5% Triton X-100, for 2 hours at room temperature. The sections were washed three times (10 minutes each wash) in DPBS, and transferred to avidin–biotin-peroxidase complex solution for 45 minutes at RT. The sections were washed three times in DPBS (10 minutes each), and the immunoperoxidase reac-

tion was developed with 3,3′-diaminobenzidine (DAB kit; SK-4100; Vector Laboratories). The slides were dried overnight, dehydrated in ethanol, cleared in xylene and cover-slipped with Entellan. All the incubations were performed in a humid chamber.

The sections were observed with a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany), and images were recorded by using a Polaroid DMC digital photocamera (Polaroid Corporation, Cambridge, MA, USA) and DMC2 software. Contrast and brightness were adjusted by using Adobe Photoshop CS3 Extended 10.0 software (Adobe Systems, San Jose, CA, USA).

Statistical analysis

Statistical analysis was carried out by using R software (http://www.R-project.org).

All the data were analysed with the Shapiro-Wilk test, to assess whether they are modelled by a normal distribution, and with the Levene test, to assess whether the variances are comparable. Normal distribution of data and homoscedasticity were assumed, with p value > 0.05.

Data were analysed through Student's t-test comparing treatments at every time point and through one-way ANOVA followed by the Tuckey post-hoc comparison to detect differences between each experimental time in a treatment group.

Results

The effects of different IM on VEGF secretion

Vascular endothelial growth factor levels (Figure 3) detected in the IM were significantly higher in DMEM + 5% FBS at D5 and D7 than in heSFM + 5% FBS (p values of 1.581×10^{-3} and 8.82×10^{-3}, respectively).

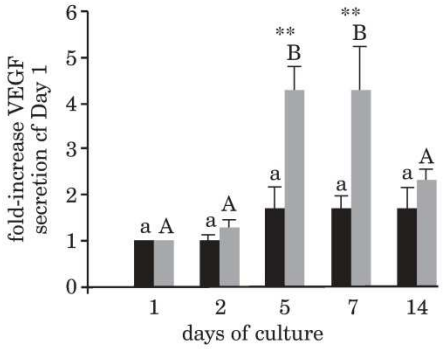
In fact, VEGF secretion was significantly increased on D5 and D7 in DMEM + 5% FBS (p value = 7.23×10^{-4}) in comparison to D1. Conversely, no statistically-significant differences were observed when the tissue was cultured in heSFM + 5% FBS (p value = 0.0908).

Activation of MMP-2 and MMP-9

Two distinct bands of gelatinase activity, corresponding to inactive MMP-2 (pro-MMP2) and active MMP-2, were evident in both tissue samples and culture media. The bands corresponding to inactive MMP-9 (pro-MMP-9) and active MMP-9 were not as evident in both the tissue samples and culture medium samples (Figure 4a).

Tissue MMP-2 (Figure 4b) undergoes activation

Figure 3: VEGF in the culture medium



■ = *heSFM + 5% FBS;* ■ = *DMEM + 5% FBS.*

*The secretion of VEGF inside the lumen of the aortic cylinder for both IM (DMEM + 5% FBS and heSFM + 5% FBS) is shown. The data are expressed as 'fold-increase' and the values were obtained by dividing the concentration of VEGF at each time-point by the concentration on Day 1. The statistical analysis is also shown. Capital letters and lowercase letters refer to one-way ANOVA analysis results, respectively, for the DMEM + 5% FBS group and the heSFM + 5% FBS group. Statistical differences were found only in the system cultured with DMEM + 5% FBS (p < 0.01). Analysis by using the Student's t-test was performed in order to compare IM at each time-point. Statistical differences were highlighted with ** (p < 0.01). The actual p value for each statistical analysis can be found in the main text.*

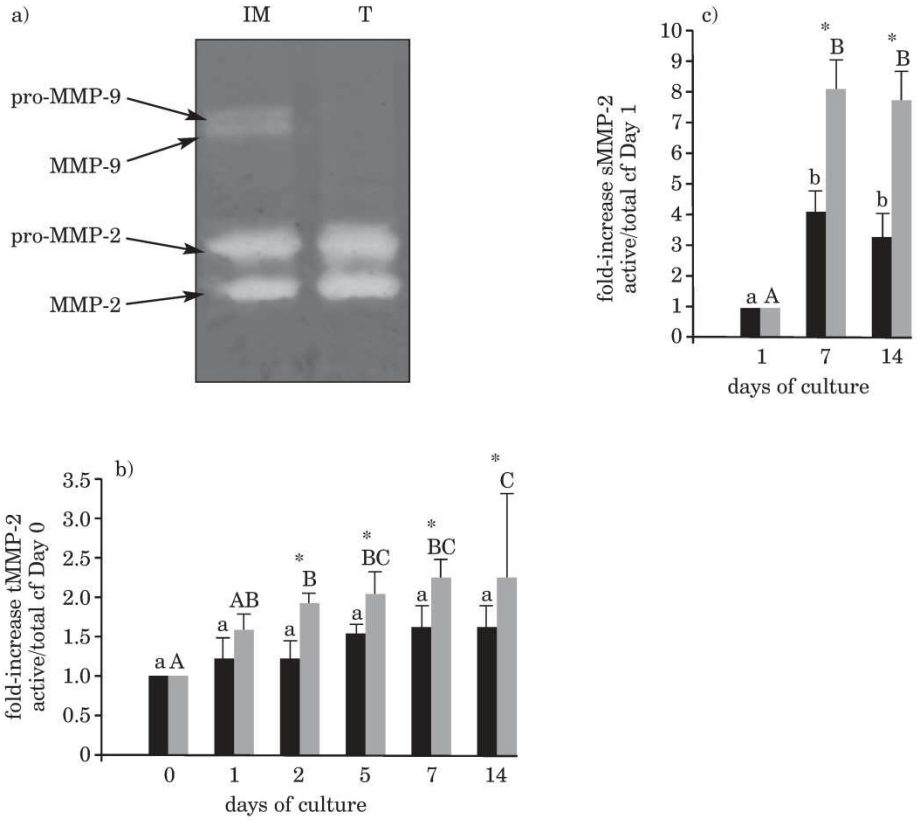
in the cut system from D2 and this activation gradually increases up to D14 (p value = 2.92×10^{-6}). Conversely, in the control system, tissue MMP-2 appears slightly activated compared to D0; however, the difference is not statistically significant (p value = 0.172).

Secreted MMP-2 (Figure 4c) was active both in the cut system (p value = 5.07×10^{-5}) and in the control aortic cylinders (p value = 0.0122), but, in particular, secreted MMP-2 activation in the injured cylinders was about two times greater than in the uncut cylinders.

Structural analysis of the aortic cylinders

The HE-stain sections showed the typical morphology of a normal porcine aortic artery. The intima, media and adventitia layers were clearly visible, with no morphological differences between the D0 and D14 samples. At both experimental time-points, the intima layer was the thinnest, whereas the media layer was the thickest (Figure 5a–d). The

Figure 4: The activation of matrix metalloproteinases during organ culture



In b) and c) = *injured (cut);* = *uninjured (control).*

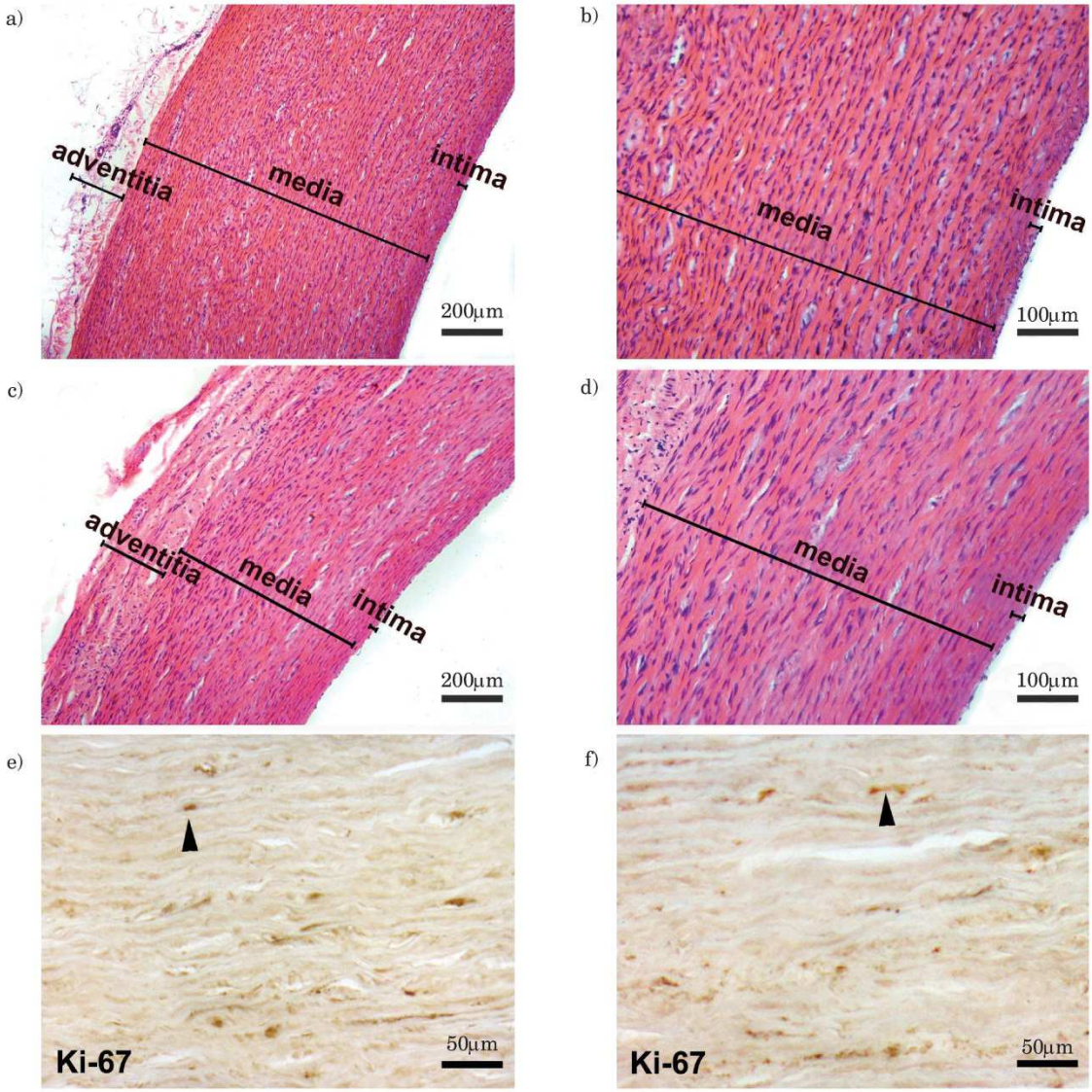
a) A representative gelatin substrate zymography gel shows gelatinase activity in tissue samples (T) and culture IM. Note that MMP-2 and pro-MMP-2 were always evident, conversely to MMP-9 and pro-MMP-9.
*b) Shows the presence of active MMP-2 in the tissue over 14 days of culture for both treatments (uninjured and injured systems); c) shows the presence of active MMP-2 in the IM over 14 days of culture for both treatments. The data were expressed as 'fold-increase' by dividing the activity of MMP-2 at each time-point by the activity at Day 1 for the media samples and at Day 0 for the tissue samples. The results of the statistical analyses are also shown. Capital letters and lowercase letters refer to the one-way ANOVA results, respectively, for injured (cut) aortic cylinders and control aortic cylinders. Significant differences were found in tissue MMP-2 activation for the injured system (*p *value < 0.01), and in secreted MMP-2 activation, both in the control (*p *value < 0.05) and cut systems (*p *value < 0.01). The actual* p *value for each ANOVA analysis can be found in the main text. Analysis by using the Student's t-test was performed in order to compare the two treatments at each time-point. Statistically significant differences (*p *value < 0.05) are highlighted in the figure with *.*

immunoreactivity for Ki-67 (Figure 5e and f), for α-smooth-muscle actin (α-SMA; Figure 5g and h) and for smooth muscle myosin (SMM) heavy-chain (Figure 5i and j), indicated by the stained cellular elements, was located especially in the intima and media layers. Interestingly, for each of the antigens examined, the distribution of immunoreactivity did not differ between D0 and D14.

Discussion

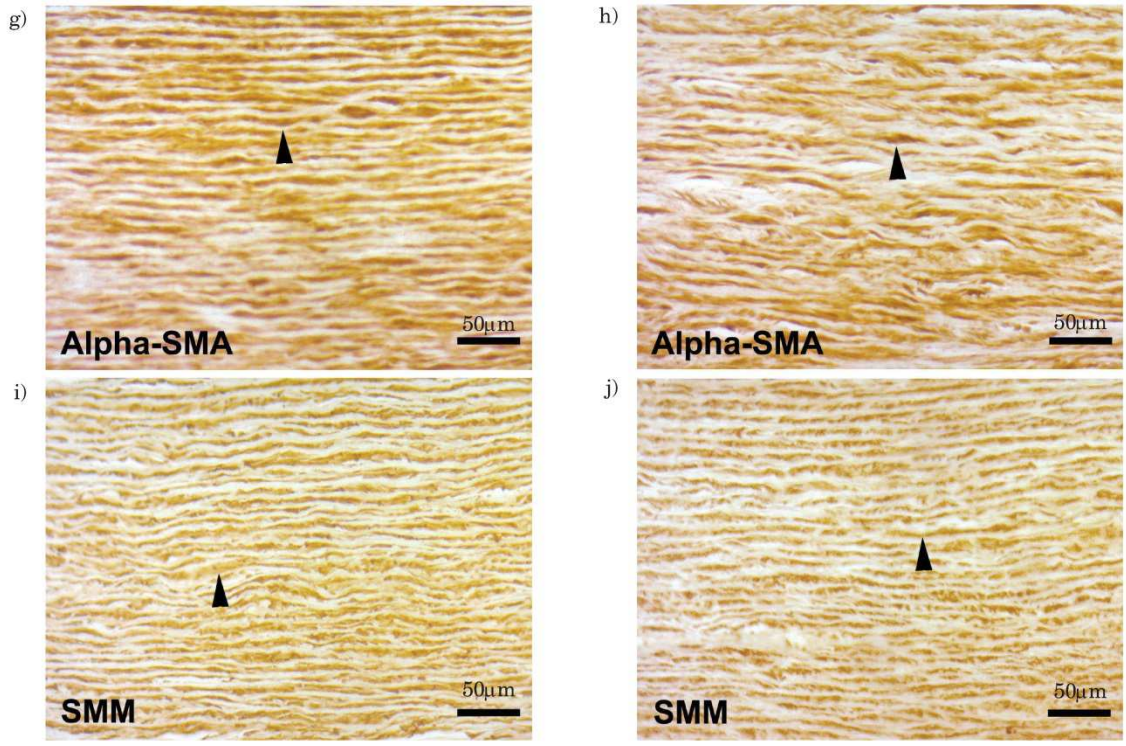
Our group has used the pig as a biomedical model for many years (3, 4, 7). In parallel with *in vivo* experiments, we aim to develop alternative methods in accordance with the principles of the Three Rs (14). In the present work, we propose a new method of aorta organ culture that permits the

Figure 5: The results from the histological analysis



Photos a to d show HE staining of the arterial wall. The intima, media, and adventitia layers exhibit a similar structure at day 0 (photos a and b), and day 14 (photos c and d). The brightfield photomicrographs of transverse section show Ki-67-immunoreactive cells (photos e and f), α-smooth muscle actin (α-SMA)-immunoreactive cells (photos g and h), and smooth-muscle myosin (SMM) heavy chain-immunoreactive cells (photos i and j), at day 0 (photos a, b, e, g and i) and day 14 (photos c, d, f, h and j). Ki-67-immunoreactivity indicates cellular proliferation; cells stained for α-SMA correspond to smooth-muscle cells and myofibroblasts; the SMM heavy-chain is expressed only in smooth-muscle cells. Immunoreactive cells are stained brown (arrowheads indicate examples of Ki-67, α-SMA, and SMM immunoreactive cells). For each antigen examined, the immunoreactivity was not different at day 0 and day 14.

Figure 5: continued



Photos a to d show HE staining of the arterial wall. The intima, media, and adventitia layers exhibit a similar structure at day 0 (photos a and b), and day 14 (photos c and d). The brightfield photomicrographs of transverse section show Ki-67-immunoreactive cells (photos e and f), α-smooth-muscle actin (α-SMA)-immunoreactive cells (photos g and h), and smooth-muscle myosin (SMM) heavy chain-immunoreactive cells (photos i and j), at day 0 (photos a, b, e, g and i) and day 14 (photos c, d, f, h and j). Ki-67-immunoreactivity indicates cellular proliferation; cells stained for α-SMA correspond to smooth-muscle cells and myofibroblasts; the SMM heavy-chain is expressed only in smooth-muscle cells. Immunoreactive cells are stained brown (arrowheads indicate examples of Ki-67, α-SMA, and SMM immunoreactive cells). For each antigen examined, the immunoreactivity was not different at day 0 and day 14.

use of different culture media for the external and internal sides of the cultured vessel.

We have characterised our organ culture model by evaluating three parameters: VEGF production, MMP activation in tissue samples and in culture media, and the maintenance of the structural integrity of the aortic wall.

VEGF is an important growth factor that is produced by several cell types, including ECs, and is released into the extracellular matrix (ECM; 25). Moreover, it is able to stimulate ECs to produce several molecules, such as MMPs, and in particular, MMP-2 and MMP-9 (26), whose main function is to degrade collagen IV in order to break down the ECM and basement membrane (BM). This step is a preliminary event in several physiological and pathological processes, such as tissue remodelling, neointimal hyperplasia, and neoplasia (27–29).

To our knowledge (23), the culture of ECs requires a specific medium for efficient growth *in vitro*. To investigate the functional stability of the endothelial layer in the aortic cylinders, we measured VEGF secretion. The results, shown in Figure 3, demonstrate that, in presence of DMEM + 10× Anti–Anti as the EM, when heSFM was used as the internal medium, VEGF secretion was not triggered during organ culture. On the contrary, when DMEM was used as the internal medium, a statistically-significant increase in the production of

VEGF at D5 and D7 of culture (p values < 0.01) was detected, which indicates an active response by ECs. The levels of VEGF observed with DMEM are comparable to those produced by primary cell cultures of porcine aortic ECs (pAECs) stimulated with bacterial lipopolysaccharide (LPS; 23). Obviously, ECs are not the only type of cells that produce VEGF, but they are the primary target of this molecule and are activated by it (29).

Although heSFM maintained the functional stability of the system for 14 days in terms of VEGF secretion, a significant increase in active secreted MMP-2 was observed (Figure 4c). Therefore, we investigated whether the edges of the cut generated when the aortic cylinders were prepared (Figure 2b), could be responsible for this increase. We performed a full depth incision on the aortic cylinder wall to generate a further cut injury. This experiment confirmed our hypothesis. In particular, our results showed that active MMP-2 was present both in the control and the injured systems, but, in the latter, the increase in active MMP-2 was significant compared to the former (Figure 4c). Secreted MMP-9 did not appear to be activated by the organ culture conditions in either system (data not shown).

These data, according to the literature, confirmed the activation of MMP-2 in mechanically-injured vessels (30), but we demonstrated that, in our system, activation occurs only near the edges of the cut.

The HE staining and the immunoreactivity of Ki-67 confirmed the overall positive structural integrity of the aortic wall after 14 days of culture (Figure 5a–f). Moreover, the immunostaining of α-SMA and SMM heavy-chain confirmed that there was no neointimal proliferation through to day 14 (Figure 5g–j). Neointimal cells typically express α-SMA, but not SMM heavy-chain (22). All of the cells in the tunica media displayed the expression of both markers, confirming their smooth muscle nature. Cells in the other layers did not display these markers.

Overall, the data obtained from the analysis of VEGF production, MMP activation, and the structural features of the tissues, indicate that the organ culture system described is stable and, in particular, that the ECs are functionally quiescent. This aorta organ culture system is an excellent experimental model for studying vascular endothelium biology, while overcoming the limitations of traditional primary EC culture. In fact, ECs in the aortic cylinder remain in their physiological context without undergoing replication. Moreover, the analysis of molecules secreted by ECs, or of molecules whose secretion is endothelium-mediated (31), in the IM, raises new and exciting perspectives in EC secretome analysis (11, 32). In addition, it could also be possible to isolate solely the endothelial layer, after *in vitro* treatment with a previously described enzymatic method (23).

This organ culture method may become an extremely important platform for studying the pathogenesis of several vascular diseases, such as atherosclerosis, hypertension and, in particular, vascular proliferative pathologies with vessel lumen stenosis (7, 8, 10). Certain pathologies, such as inflammation, could be studied at the molecular level with the proposed system, and gene therapy (33) and molecular therapy of diseases, such as the down-regulation of genes involved in pathogenesis (34), could be developed as a means of testing new biotechnological drugs.

To conclude, we have characterised our proposed endothelial vessel organ culture system for three key specific parameters, and have shown that it exhibits properties similar to those of *in vivo* tissue. The model can contribute to a reduction in the numbers of experimental animals used in research on vascular endothelium physiology and the pathogenesis of vascular diseases. We chose to develop this method by using porcine vessels, because the pig is recognised as an excellent model for cardiovascular disease (5, 6), and porcine aortas can be collected both in surgical theatres and at slaughterhouses, without sacrificing any animals specifically for the purposes of the research. In the former case, our method leads to a relative replacement, and, in the latter, it leads to a true reduction of the number of animals used for experimental purposes.

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THE DOROTHY HEGARTY AWARD

This year's Dorothy Hegarty Award has been won by Andrea Zaniboni, Augusta Zannoni, Chiara Bernardini, Cristiano Bombardi, Eraldo Seren, Monica Forni and Maria L. Bacci (Department of Veterinary Medical Sciences — DIMEVET, University of Bologna, Bologna, Italy) and Marco De Cecco (Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI, USA).

Their paper, Development of a Vessel Organ Culture System: Characterisation of the Method and Implications for the Reduction of Animal Experiments, appeared in ATLA 41, pp. 259–269.

The Award is presented annually to the authors of the paper published in the previous year's volume of FRAME's scientific journal, ATLA, which, in the opinion of the members of the Editorial Board, is likely to make the most significant contribution to the reduction, refinement and/or replacement of animal experimentation.

Each member of the ATLA Editorial Board is entitled to make up to five nominations for the Award, in rank order. As in previous years, a large variety of papers were nominated, reflecting the diversity of the work published in ATLA, and the wide range of interests of the members of the Editorial Board.

Here, the authors have summarised the importance of their paper to the progress of Three Rs:

"Our research group has used the pig as a biomedical preclinical model for many years. However, the use of this species is really complex, especially because it is less standardisable, as compared to the mouse and the rat. Despite this, the pig has a great value in preclinical studies due to its great similarity to humans (e.g. its genome, anatomy, physiology), especially for research on cardiovascular physiopathology.

"The greatest reduction in experimental animal numbers could be achieved by collecting a large amount of preliminary data from in vitro and ex vivo studies, prior to performing a preclinical in vivo study. In view of this, with a grant from Fondazione del Monte di Bologna e Ravenna, our group developed an ex vivo organ culture system of the pig aorta that could overcome some of the limitations of primary cell cultures (i.e. lack of physiological context). With this method, we aimed to study restenosis/neointimal development and formation and associated molecular biology.

"Our model allows researchers to study vascular processes without the sacrifice of experimental animals, because porcine aortas can be recovered at the slaughterhouse or from control animals sacrificed for other experimental purposes. We really believe that our model has contributed to an improvement in the panel of alternative methods available to study vascular physiopathology, according to the Three Rs principles."

DEVELOPMENT OF A PRIMARY CULTURE OF MESENCHYMAL-LIKE STROMAL CELLS DERIVED FROM PORCINE AORTA

Mesenchymal Stromal Cells (MSCs): a brief history

Mesenchymal Stem Cells (MSCs) were described for the first time by Friedstein and colleagues as bone marrow derived progenitor cells. In their papers the authors described a subpopulation of bone marrow – nonhemopoietic clonogenic cells able to rapidly adhere to plastic with a fibroblast-like morphology (CFU-Fs – colony forming unit-fibroblasts). These cells owned an *in vivo* osteogenic potential showed through cell transplantation experiments (Friedenstein et al., 1968, 1970, 1974; Owen & Friedenstien, 1988; Keating, 2012). Their osteogenic potential *in vivo* corresponds to the possibility to generate, in an heterotopic location, a new-formed ossicle in which all skeletal tissues (bone, cartilage, adipose tissue and fibrous tissue) can be recognized (Uccelli et al., 2008; Bianco et al., 2008; Bianco et al., 2013; Frenette et al., 2013). These cells were further defined osteogenic stem cells (Friedenstein et al., 1987). Osteogenic stem cells were shown to be able to differentiate towards the adipo-, chondro- and osteocytic lineages not only *in vivo*, but also *in vitro* (Pittenger et al., 1999).

Based on the evidence of *in vivo* multipotency and on the suggestion of self-renewal of the bone marrow derived stem cells, Caplan (1991) coined the term “Mesenchymal Stem Cells” (Caplan, 1991).

In the 1990s and 2000s a plethora of papers concerning the isolation of the so-called “Mesenchymal Stem Cells” from different tissues (not only from the bone marrow) and with different methods have been published. As such, a significant debate concerning the requirement of new terminologies and different criteria to better define these cells started to grow among scientific community (Keating et al., 2012).

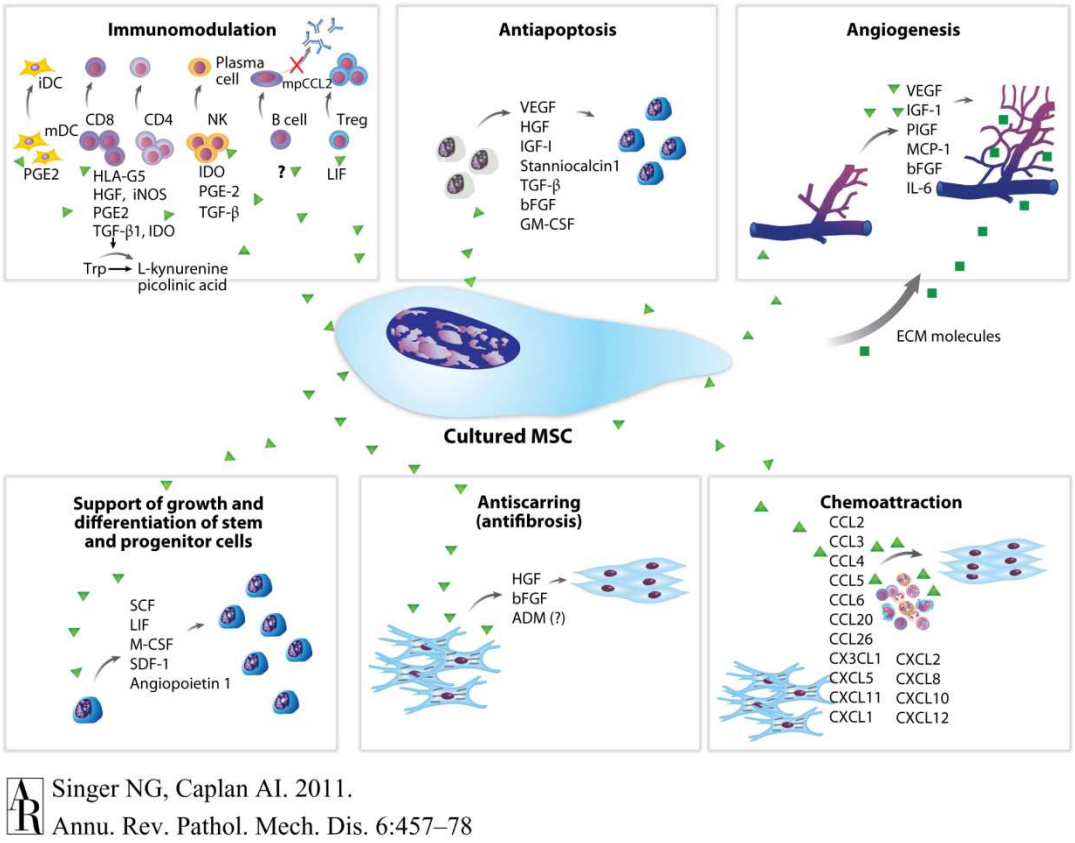
In order to better clarify and define these points, the International Society for Cellular Therapy published (ISCT) two position papers. (Horwitz et al., 2005; Dominici et al., 2006).
First of all, the authors invite the reader to refer to the most of these cells as multipotent “Mesenchymal Stromal Cells – MSCs” (due to their tissues of origin).

The term "Mesenchymal Stem Cells", they argue, is only to use when the stem cell properties are clearly demonstrated (Horwitz et al., 2005; Dominici et al., 2006).
On this subject Bianco and colleagues stated that, in order to define MSCs as "stem", a precise assessment of the self-renewal and of the multilineage potential is needed (Bianco et al., 2008).
However, the overlap between the acronyms used for both the mesenchymal stromal and stem cells (MSCs) may result confusing. Thus, from now on in the paper the acronym MSCs will be used only to refer to multipotent Mesenchymal Stromal Cells.

The ISCT also defined some fundamentals criteria in order to define MSCs: 1) these cells have to adhere to plastic in standard culture conditions, 2) they have to express CD105 (endoglin), CD90 (Thy-1 - Thymocyte differentiation antigen 1), CD73 (ecto-5'-nucleotidase) surface markers and lack the expression of the hemopoietic markers 3) they have to differentiate *in vitro* towards the adipo-, osteo- and chondrocytic phenotypes (Horwitz et al., 2005; Dominici et al., 2006).

Many studies have already described that, nowadays, it exists the possibility to isolate MSCs populations from adipose tissue, placenta, umbilical cord blood, Wharton's Jelly, dental pulp, amniotic fluid, synovial membranes and other tissues (Singer & Caplan, 2011; Keating et al., 2012; Murray et al., 2014).

In bone marrow the function of Mesenchymal Stem Cells is to support hemopoiesis and to stabilize and to help the development of sinusoidal network (Sacchetti et al., 2007; Kunisaki et al., 2013; Frenette et al., 2013).
Sacchetti and colleagues (2007) demonstrated for the first time that CD146 (MCAM –Melanoma Cell Adhesion Molecule) positive clones of Mesenchymal Stem Cells, derived from bone marrow stroma, are able to re-generate an hemopoietic microenviroment if transplanted *in vivo* into an heterotopic site (Bianco et al., 2008; Bianco et al., 2013; Frenette et al., 2013). The function of the bone marrow Mesenchymal Stem Cells in the hemopoietic niche was also properly reviewed by Frenette and colleagues (2013).



Singer NG, Caplan AI. 2011.
Annu. Rev. Pathol. Mech. Dis. 6:457–78

Figure 1: *Paracrine mechanisms of action of MSCs. MSCs secrete a broad range of bioactive molecules and growth factors which are essential in immunomodulation, apoptosis, angiogenesis, maintenance of hemopoietic niche, antiscarring processes and chemoattraction. The immunomodulary effect is achieved through the inhibition of both innate and adaptive immune mechanisms secreting molecules able to inhibit proliferation of CD4+ and CD8+ lymphocytes and natural killer cells, to suppress the production of immunoglobulin as well as to inhibit the maturation of dendritic cells and to stimulate the proliferation of regulatory T cells. Anti-apoptotic mechanism is guided by growth factors such as: Hepatocyte Growth Factor (HGF), Transforming Growth Factor (TGF)-β, Vascular Endothelial Growth Factor (VEGF), Insulin-like Growth Factor (IGF)-1 and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF). VEGF and IGF-1 are involved in the pro-angiogenic effect of MSCs with other extracellular matrix (ECM) molecules, basic Fibroblast Growth Factor (bFGF) and interleukin (IL)-6. Moreover, HGF and bFGF contribute to the inhibition of scarring. MSCs stimulate mitosis of hemopoietic progenitors secreting Stem Cell Factor (SCF), Macrophage Colony–Stimulating Factor (M-CSF), Stromal cell–derived Factor (SDF-1), Leukemia Inhibiting Factor (LIF) and angiopoietin 1. Finally, the chemoattraction of leukocyte to a site of injury is also driven by MSCs through the secretion of chemokines (Singer & Caplan, 2011).*

Regenerative medicine is likely to be the major field of therapeutic application of MSCs (Frenette et al., 2013). Within regenerative medicine, the clinical interest of MSCs resides in their documented paracrine properties.

It is thought, that through soluble factors release, MSCs are able to carry out their therapeutic functions. Trophic and immunomodulatory molecules released by MSCs are involved in immunomodulation, apoptosis, angiogenesis, hemopoietic niche maintenance, fibrosis and chemoattraction (da Silva Meirelles et al., 2009; Singer and Caplan, 2011) as shown in Figure 1.

MSCs secretome came into focus in several research fields, among which the development of cell therapy for cardiovascular diseases (i.e. for ischemic heart disease). A better and more exact characterization of secreted cytokines and trophic factors would help in understanding the therapeutic effects of these cells (Ranganath et al., 2012).

Perivascular Cells and Vascular Stem Cells (VSCs)

The demonstration that Mesenchymal Stem Cells in the bone marrow sinusoids are perivascular cells (Sacchetti et al., 2007; Bianco et al., 2013) led scientists to look for the niche of those MSCs derived from the other tissues of the organism. MSCs are, in fact, the most elusive population of adult multipotent cells (Murray et al., 2014).

According to the copious number of papers stating that MSCs may be isolated from almost all vascularized organs, some authors define perivascular cells (aka mural cells) as the possible source of the ubiquitous MSCs *in vivo* (Murray et al., 2014). Conversely, other authors claim that these mural cells are just tissue specific progenitors, with specific differentiation potential, and that further studies are needed in order to clearly define their features (Bianco et al., 2013).

Despite the ongoing scientific debates regarding nomenclature, source, *in vivo* location and differentiation potential of mesenchymal stem/stromal/progenitor cells, it's now been several years since the presence of progenitor cells within the vessel wall was described for the first time (Tintut et al., 2003; Pacilli & Pasquinelli, 2009; Psaltis et al., 2011; Chen et al., 2012; Murray et al., 2014).

A lot of work has indeed been done in order to characterize and describe these populations of perivascular cells, although it has been reported that a prospective approach might help in isolating them (Chen et al., 2012; Corselli et al., 2013 b; Murray et al., 2014).

In particular, three main cell types were isolated through prospective purification: myoendothelial cells (MECs) (Zheng et al., 2007), pericytes (PCs) (Crisan et al., 2008) and advential cells (ACs) (Corselli et al., 2012).

Prospective isolation is based on an initial *in vivo* characterization of the cells. This procedure is performed through immunodetection in order to find the few surface markers whose combination univocally identifies the cell type of interest. Then,

using a Fluorescence Activated Cell Sorting (FACS) approach, it is possible to isolate pure populations of mural cells for the co-expression of those surface markers and the lack of the unspecific ones.

Basically, tissues are digested by a collagenase treatment and then monocellular suspension is stained with anti- CD146 (MCAM), CD34, CD56 (NCAM – Neural Cell Adhesion Molecule), CD45 (PTPRC – Protein Tyrosine Phosphatase, Receptor type, C), CD31 (PECAM – Platelet Endothelial Cell Adhesion Molecule) or CD144 (Vascular Endothelial (VE)-Cadherin) antibodies. The perivascular cells from intima, media and adventitia layers of the vessel wall are then sorted according to the marker combinations shown in Figure 2 (Chen et al., 2012; Corselli et al., 2013 b; Murray et al., 2014). Figure 2 describes differentiation potential, both *in vivo* and *in vitro*, of perivascular cells and their potential preclinical application (Chen et al., 2012).

	MEC	Pericyte	AC	BM-MSC
Native location	Intima	Media	Adventitia	Bone marrow
Cell surface marker profile for cell sorting	CD34+ CD45− CD56+ CD144+	CD34− CD45− CD56− CD146+	CD31− CD34+ CD45− CD146−	N/A
Classic MSC marker expression in culture	CD29+ CD44+ CD90+ CD105+	CD44+ CD73+ CD90+ CD105+	CD44+ CD73+ CD90+ CD105+	CD29+ CD44+ CD73+ CD90+ CD105+
Differentiation *in vitro*	Osteogenic (+) Chondrogenic (+) Adipogenic (ND) Myogenic (+)	Osteogenic (+) Chondrogenic (+) Adipogenic (+) Myogenic (+)	Osteogenic (+) Chondrogenic (+) Adipogenic (+) Myogenic (ND)	Osteogenic (+) Chondrogenic (+) Adipogenic (+) Myogenic (+)
Differentiation *in vivo*	Myogenesis	Myogenesis Osteogenesis	Vasculogenesis	Osteogenesis Chondrogenesis Adipogenesis Myogenesis Cardiomyogenesis Vasculogenesis
Potential clinical application	Skeletal musclere-pair/regeneration; Cardiac repair	Skeletal muscle repair/regeneration; Vascular repair/regeneration	Vascular repair/regeneration; Cardiac repair	Bone repair; Cartilage repair; Tendon/ligament repair; skeletal muscle repair; Vascular repair; Cardiac repair; Wound healing; Immunoregulation

MEC: myogenic endothelial cell; AC: adventitial cell; BM-MSC: bone marrow mesenchymal stem/stromal cells; N/A: not available; ND: not determined.

Figure 2: *In the present table myoendothalial cells (MEC), pericytes and adventitial cells (AC) immunophenotype, differentiation potential, both in vivo and in vitro, and potential clinical application was reported by Chen and colleagues (2012) (Chen et al., 2012).*

The potential of pericytes in regenerative medicine found its targets in the treatment of myocardial ischemia (Chen et al., 2013; Chen et al., 2015), peripheral ischemia (Dar et al., 2012) and bone regeneration (James et al., 2012).
Moreover, recent data demonstrated that CD146 positive perivascular cells support hemopoietic stem/progenitor cells via cell-cell interactions (Corselli et al., 2013 a).

Perivascular cells, as described by Chen and colleagues (2012), are not the only population of cells identified within the vessel wall. In their work, Psaltis and colleagues (2011) review in detail the bibliography related to mural and perivascular cells describing mesangioblasts, vascular smooth muscle precursors and progenitors, endothelial progenitors and pericytes, as well as their involvement in physio-pathological processes (Psaltis et al., 2011; Chen et al., 2012).

A recent review by Lin and Lue (2013) properly describes a series of vascular wall resident multipotent cells among which the so-called Vascular Stem Cells (VSCs). The authors state that VSCs indeed correspond to MSCs due to their trilineage differentiation potential and due also to their ability to differentiate towards the vascular smooth muscle and endothelial phenotypes. Pericytes and adventitial cells are recognized, by the authors, as the most plausible VSCs population within the vessel wall (Lin & Lue, 2013).

Perivascular cells, as well as vascular resident multipotent cells, can also be involved in vascular pathology (Hu & Xu, 2011). In particular, Tang and colleagues (2012) demonstrated that multipotent vascular stem cells are involved in neointimal hyperplasia in restenosis (Tang et al., 2012).

Porcine multipotent mesenchymal stromal cells

Porcine MSCs (pMSCs) have already been isolated and described (Casado et al., 2012). Nonetheless, due to the lack of anti-pig specific antibodies and of cross-reactivity with some anti-human antibodies, their proper phenotypical characterization is quite difficult (Rozemuller et al., 2010). However, the isolation and characterization of pMSCs have been carried out anyway in order to develop translational models for regenerative medicine/cell transplantation purposes.

Comite and colleagues (2010) described the isolation of pMSCs from bone marrow. Cells were isolated depending on their adhesion properties to plastic support and characterized for the expression of CD105, CD90 and CD29 (Integrin beta-1) and the lack of the expression of CD45 and CD11b (Integrin alpha M) (Comite et al., 2010). Moreover, bone marrow derived pMSCs displayed an osteogenic and adipogenic potential *in vitro*.
Peterbauer-Scherb and colleagues (2010) induced chondrogenic differentiation of bone marrow derived pMCSs with the aim to utilize these cells in porcine models of regenerative medicine within the orthopedic field (Peterbauer-Scherb et al., 2010).

A comparison between bone marrow derived human MSCs (hMSCs) and pMSCs was carried out by Noort and colleagues (2012). From the phenotypical point of view, these species share the expression of the surface markers CD90, CD44 (Homing Cell Adhesion Molecule – HCAM), CD29, CD271 (Nerve Growth Factor Receptor – NGFR), CD146, CD56 and the lack of the expression of CD34, CD45, CD14 and HLA (Human Leukocyte Antigen)/SLA (Swine Leukocyte Antigen) class II. Trilineage differentiation potential was demonstrated for both hMSCs and pMSCs. Injection of hMSCs and pMSC in murine model of myocardial ischemia resulted in improvement of ejection fraction compared to PBS injection. These data confirmed the usefulness of the pig model in cell therapy-based regenerative medicine (Noort et al., 2012).

Recently, Brückner and colleagues (2014) demonstrated the hepatocytic differentiation of bone marrow and adipose derived pMSCs. Differentiated cells were morphologically and functionally characterized in order to validate the pig model to study liver physio-pathology, as well (Brückner et al., 2014).

All these data confirm the importance and the relevance of the pig in cell based regenerative medicine, as widely described in the previous chapter "*Involvement of the porcine model in regenerative medicine*".

Isolation of MSC-like cells from porcine aorta: purposes and papers

As stated in the aim chapter, two papers were produced with the data obtained from this research.
The first paper was published in the *American Journal of Cell Physiology (AJP) – cell physiology* (Zaniboni et al., 2014) and pertains the establishment of a new method for the isolation of MSC-like cells derived from porcine aorta media layer.
The cells were properly described and characterized in the first paper. The same were then cultured in a perivascular cells specific medium. Thus, the differentiation potential of these cells towards the cell phenotypes constituting the vascular wall was assessed in order to evaluate their VSCs properties. These results were then drafted in another article that is currently submitted for publication in *AJP – cell physiology*.

Considering the importance of the vascular wall resident MSCs in fields of regenerative medicine (Chen et al., 2102; Lin & Lue, 2013), the main purpose of these works was to develop a new, well characterized and useful *in vitro* tool to study the vascular biology and the pathogenesis of cardiovascular diseases (Hu & Xu, 2011; Tang et al., 2012).

The *in vitro* system developed in this research led to a reduction and a refinement of animal experimentation because aortas could be recovered from animals killed at slaughterhouse or from control animal sacrificed for other experimental purposes.

Development of the method and characterization of cells

The isolation of the cells was carried out by modifying a method previously published by the BCM laboratory (Bernardini et al., 2005), routinely used for the isolation of porcine Aortic Endothelial Cells (pAECs).
The new method is based on the collagenase digestion of the porcine aorta from the inner side of the vessel. After forty minutes of digestion of the vessel wall, cellular suspension is recovered (endothelial cell fraction) and the aorta is filled with collagenase solution, again. After other four hours of digestion, cellular suspension is newly recovered, then seeded in a cell culture flask and cultured overnight in an high antibiotic-antimycotic medium. After three days of culture in a standard culture medium (DMEM with 10% Fetal Bovine Serum – FBS) cells are serum starved and then routinely cultured and expanded.

For the purposes of the work, Passage 3 (P3) cells were characterized for surface marker expression, trilineage differentiation potential and for their pericyte-like properties in supporting angiogenesis.

Results obtained from the histological analysis of the vessel wall revealed that, after forty minutes of collagenase digestion, the intima layer is completely lost and, after four hours, about one third of the media layer is digested. These results suggested a media layer *in vivo* localization of the isolated cells.

The phenotypical characterization revealed that cells derived from porcine aorta media layer expressed, *in vitro*, several MSC-pericyte markers.
In particular, immunocytochemical analysis revealed the expression of vimentin, laminin, nestin, Platelet Derived Growth Factor Receptor (PDGFR)-α, PDGFR-β, Neural Glial antigen-2 (NG2) and α-Smooth Muscle Actin as described for cultured pericytes (Howson et al., 2005; Crisan et al., 2008). No expressions of smooth muscle (smooth muscle myosin-heavy chain) and endothelial cell (PECAM aka CD31) markers were detected.

Immunophenotyping of cells revealed, instead, the expression of CD105, CD90, CD44 and the lack of expression of CD45 and CD34 as requested for MSCs (Dominici et al., 2006). Moreover, as already described for sub-population of MSCs by several authors, (Battula et al., 2009; Bühring et al., 2009; Rossignoli et al., 2013) the expression of CD56 were shown for cells derived from porcine aorta media layer. Besides these phenotypical data, gene expression analysis through PCR revealed the presence of CD105, CD90, CD73 transcripts.

Trilineage differentiation potential was also shown for cells derived from porcine aorta media layer that differentiated towards the adipo-, osteo- and chondrocytic phenotypes, as requested for MSCs (Dominici et al., 2006).

Lastly, through an *in vitro* angiogenesis assay, based on the co-culture with Human Umbilical Vein Endothelial Cells (HUVEC) seeded on extracellular matrix, it was demonstrated the support of the cells derived from porcine aorta media layer to the angiogenic process. This is considered a particular feature of pericytes (Blocki et al., 2013).

Taking into account all the results obtained from the analysis of the cells derived from porcine aorta media layer it is possible to conclude that:

- the method hereby described is simple and suitable as it allows to isolate a predominant population of cells from porcine aorta media layer;
- these cells share phenotypical and differentiative features with MSCs;
- these cells share phenotypical and functional features with pericytes.

All these considerations led to define these cells as porcine Aortic Precursor Cells (pAVPCs) due to their MSC/pericyte-like nature.

Article

Unfortunately, the full-text article cannot be published in the electronic version of this thesis due to *AJP* copyright policy.
However, the reader can find the paper using the following reference:

Zaniboni A, Bernardini C, Alessandri M, Mangano C, Zannoni A, Bianchi F, Sarli G, Calzà L, Bacci ML, Forni M. **Cells derived from porcine aorta tunica media show mesenchymal stromal-like cell properties in in vitro culture.** *Am J Physiol Cell Physiol* 2014;306(4):C322-33. (PMID: 24304832)

Abstract

(*see the screenshot from PubMed website below*)

Am J Physiol Cell Physiol. 2014 Feb 15;306(4):C322-33. doi: 10.1152/ajpcell.00112.2013. Epub 2013 Dec 4.

Cells derived from porcine aorta tunica media show mesenchymal stromal-like cell properties in in vitro culture.

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Author information

Abstract

Several studies have already described the presence of specialized niches of precursor cells in vasculature wall, and it has been shown that these populations share several features with mesenchymal stromal cells (MSCs). Considering the relevance of MSCs in the cardiovascular physiopathology and regenerative medicine, and the usefulness of the pig animal model in this field, we reported a new method for MSC-like cell isolation from pig aorta. Filling the vessel with a collagenase solution for 40 min, all endothelial cells were detached and discarded and then collagenase treatment was repeated for 4 h to digest approximately one-third of the tunica media. The ability of our method to select a population of MSC-like cells from tunica media could be ascribed in part to the elimination of contaminant cells from the intimal layer and in part to the overnight culture in the high antibiotic/antimycotic condition and to the starvation step. Aortic-derived cells show an elongated, spindle shape, fibroblast-like morphology, as reported for MSCs, stain positively for CD44, CD56, CD90, and CD105; stain negatively for CD34 and CD45; and express CD73 mRNA. Moreover, these cells show the classical mesenchymal trilineage differentiation potential. Under our in vitro culture conditions, aortic-derived cells share some phenotypical features with pericytes and are able to take part in the formation of network-like structures if cocultured with human umbilical vein endothelial cells. In conclusion, our work reports a simple and highly suitable method for obtaining large numbers of precursor MSC-like cells derived from the porcine aortic wall.

KEYWORDS: mesenchymal stromal cells; perivascular cells; porcine animal model

Differentiation potential through the vascular smooth muscle and the endothelial phenotypes

As previously described, porcine Aortic Precursor Cells have been properly characterized as MSC/pericyte-like cells for surface markers expression, trilineage differentiation potential and their support to angiogenesis *in vitro* (Zaniboni et al., 2014).

Cells were cultured in a standard culture medium constituted of Dulbecco's Modified Eagle Medium (DMEM) and Medium 199 (M199) (ratio 1:1) added with 10% of Fetal Bovine Serum (FBS) with 1% of antibiotic – antimycotic (DM medium). Despite the characterization results, it has been observed that cells cultured in DM medium undergo towards the loss of multipotency and towards the so-called "cellular senescence" due to several passages *in vitro* (observation).

In order to overcome the limits of the *in vitro* culture with the DM medium, the protocol herby described was modified with the purpose of culturing pAVPCs in a pericytes specific medium (Pericytes Growth Medium, Promocell – PGM).

MSC/pericyte properties of pAVPCs cultured in PGM were then assessed and, moreover, the differentiation towards the vascular smooth muscle and the endothelial cell phenotypes was evaluated, too.

The change of culture conditions involved an equivalent one in cell morphology. Cells cultured in DM medium displayed a fibroblast-like morphology (as described for MSCs – Dominici et al., 2006), while cells cultured in PGM displayed a small cell body with little thin arms (as described for pericytes – Crisan et al., 2008). Moreover, cells cultured in PGM grew more rapidly than cells cultured in DM medium; doubling time is reduced of about 10 hours among DM culture and PGM culture (Zaniboni et al., 2014).

Passage 3 pAVPCs were characterized for the expression of MSC/perivascular cell transcripts through quantitative Polymerase Chain Reaction (qPCR). The expression of CD105, CD90, CD73, CD56 and the lack of CD45 transcripts were shown for PGM cultured pAVPC, as for MSCs (Dominici et al,. 2006, Rossignoli et al., 2013). Nonetheless, PGM cultured cells expressed transcripts of NG2, Nestin, CD146, αSMA, PDGFRβ, which are considered the main pericytes markers (Crisan et al., 2008), and CD34 transcript, which is considered a marker of adventitial cells and myoendothelial cells (Corselli et al., 2012). Both transcripts of the growth factor VEGF and PDGFβ were also detected as well as the expression of VEGF receptor (VEGFR)-1 and VEGFR-2.

Moreover, the work also investigated phenotypical features of PGM cultured cells.
Passage 3 pAVPC expressed CD105 (66.8 ± 0.1 %), CD90 (99.5 ± 0.2 %), CD44 (99.6 ± 0.3 %) and lacked (less than 2%) the expression of CD45 (1.4 ± 0.4 %), CD34 (1.3 ± 0.1 %), as requested for MSCs characterization (Dominici et al., 2006). To exclude possible endothelial cells contaminations, cells were analyzed for the expression of CD31 and just 1.5 ± 0.1 % of them expressed it. Moreover, cells expressed CD56 (99.9 ± 0.1 %) an MSC-subset marker (Battula et al., 2009; Bühring et al., 2009; Rossignoli et al., 2013).
Immunocytochemistry revealed the expression of PDGFRβ, NG2 and Nestin pericyte markers and the lack of expression of αSMA (less than 2%). The latter is considered a differentiation marker for multipotent pericytes. It is present in fully differentiated functional pericytes, instead it lacks in the multipotent ones. Conversely to the results obtained for the DM culture, the lack of expression of αSMA indicates that PGM is able to maintain cells in an uncommitted status (Crisan et al., 2012). Cells do not express CD34 and CD31 proteins.
Results obtained from the PGM cultured pAVPC phenotypical analysis revealed a more uniform population of MSC/pericytes-like cells, compared to the culture in DM (Zaniboni et al., 2014).

Mesenchymal Stromal Cells *in vitro* trilineage differentiation potential (Dominci et al., 2006) was also evaluated for PGM cultured pAVPCs. After adipo-, osteo-,

chondrogenic differentiation induction, qPCR results showed the expression of typical markers of fully differentiated adipocytes, osteocytes and chondrocytes, respectively. Moreover, histological analysis revealed lipid droplets, within the cells bodies, in adipo-differentiated cells, calcium deposits in osteo-differentiated cells and proteoglicans in chondro-differentiated cells.

To assess the spontaneous differentiation towards the vascular smooth muscle phenotype (Tang et al., 2012), pAVPCs were cultured for 21 days in DMEM + 10% FBS (long term medium – LTM). Quantitative PCR analysis revealed an overexpression of αSMA, calponin (CNN1) and smooth muscle myosin heavy chain (SMM-hc) transcripts (Xie et al., 2011). Immunocytochemistry revealed the expression of αSMA and SMM in LTM cultured cells compared to control. These data confirmed that pAVPCs are able to spontaneously differentiate to vascular smooth muscle cells as already described by Tang and colleagues (2012) for other multipotent vascular cells.

To assess the differentiation potential of PGM cultured cells towards the endothelial cell phenotype, cells were cultured for 21 days in an endothelial cell medium (EDM medium), supplemented with VEGF. Quantitative PCR analysis revealed an overexpression of CD31, vascular endothelial(VE)-Cadherin, von Willebrand Factor, endothelial nitric oxide synthase (eNOS) transcripts (Xie et al., 2011). Moreover, immunocytochemistry revealed the expression of CD31 in EDM cultured cells compared to control. These data confirmed that pAVPCs are able to differentiate towards the endothelial cell phenotype, as already described for porcine bone marrow-derived MSCs by Pankajakshan and colleagues (2013).

Taken together, these results confirmed that cells cultured in PGM possess MSC/pericyte properties (Dominici et al. 2006) and that they display VSC-like features according to the definition given by Lin and Lue (2013) of Vascular Stem Cells (Lin & Lue, 2013).

In particular, culturing pAVPCs in PGM allowed the isolation of a uniform population of mesenchymal cells which possessed trilineage differentiation potential, vascular smooth muscle and endothelial differentiation potential. Nonetheless, it is worth to remember that the pro-angiogenic properties of pAVPCs cultured in PGM had already been demonstrated (Zaniboni et al., 2014) through an *in vitro* angiogenesis assay with HUVEC.

The method hereby described offers important starting points to the purposes of regenerative medicine considering the peculiarity of these cells to differentiate towards the endothelial lineage (Dar et al., 2012; James et al., 2012; Chen et al., 2013). It is also equally important to underline the spontaneous ability of the same cells to differentiate towards the vascular smooth muscle cell phenotype and so, their possible involvement in vascular diseases pathogenesis, as already described for other vascular derived multipotent cell types (Juchem et al., 2010; Hu & Xu, 2011; Tang et al., 2012).

In conclusion, according to the recent Lin & Lue review, it is possible to define porcine Aortic Vascular Precursor Cells as a population of Vascular Stem Cell-like cells.

Indeed, when considering the importance of the porcine model within the cardiovascular research field, further investigation are required in order to define these cells possible involvement in vascular pathology and regenerative medicine.

Submission to AJP-Cell Physiology

Andrea Zaniboni

Da: edwyer@the-aps.org
Inviato: mercoledì 18 febbraio 2015 20:08
A: Andrea Zaniboni
Oggetto: C-00049-2015 Receipt of New Paper by American Journal of Physiology - Cell Physiology

Dear Dr. Zaniboni,

On 18th Feb 2015, I received your manuscript entitled "In vitro differentiation of porcine Aortic Vascular Precursor Cells to Endothelial and Vascular Smooth Muscle cells" by Andrea Zaniboni, Chiara Bernardini, Martina Bertocchi, Augusta Zannoni, Francesca Bianchi, Giancarlo Avallone, Chiara Mangano, Giuseppe Sarli, Laura Calzà, Maria Laura Bacci, and Monica Forni.

Your manuscript has been assigned the Paper #: C-00049-2015.

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Thank you for submitting your work to the American Journal of Physiology - Cell Physiology.

Sincerely,

Paul Insel
Editor-in-Chief
AJP-Cell Physiology

***Confirmation email**: please note that the web URL to access the paper page has been modified in order to avoid any inconvenience with the online publication of the thesis.*

As said above, the paper describing the vascular smooth muscle and the endothelial differentiation of pAVPCs is currently submitted for review to the *AJP-Cell Physiology* (*see confirmation email above*).

Although it is not possible to insert the whole work into the present thesis, due to publication policy, the previous pages describe in detail its full content and its most important remarks.

CONCLUDING REMARKS

The present manuscript reports an attempt to expand the range of already existing *ex vivo* and *in vivo* methods to study cardiovascular biology.
With the Directive 2010/63/EU, the European Union, asked the scientific community to establish, characterize and validate new alternative methods to animal experimentation for translational research.

The choice of the porcine species as the subject of the research is based on the continuous evidence that the pig is the most similar non-primate animal model to the human species. Moreover, the pig has been shown to be a valuable animal model within the cardiovascular research field both for physiology and pathology.
There are several wild-type and transgenic porcine models generated for biomedicine and translational research fields of study. Among these, the cardiovascular ones are indeed the most represented.

This work was then based on the development of two models which take into account Russell and Burch's 3Rs (Reduction, Refinement, Replacement). In particular, both the models led to a Refinement and to a Reduction of animal experimentation.

The first method consists in an organ culture of porcine aorta developed with the aim to supply the scientific community with a new tool in vascular biology research field. Due to the fact that the model consists in the culture of portions of the aorta it resembles the *in vivo* physiological condition more than primary cell culture of endothelial cells does.
The results obtained from the histological and molecular characterization of the method proved not only that it closely mimes the *in vivo* conditions, but also that it is a stable system, at least for fourteen days of culture. The most immediate application of the aorta organ culture is its use in the study of vascular restenosis and more widely in the study of vascular physio-pathology.

The second method consists in a primary *in vitro* culture of precursor cells derived from porcine aorta, developed with the aim to provide a new tool within the fields of

cell-based translational medicine and vascular pathology. This new tool was thought up in order to supply the scientific community with a simpler and more suitable method allowing the isolation of a large number of Mesenchymal Stromal/Vascular Stem Cell-like cells.
Precursor cells, isolated from the pig aorta, were characterized for surface markers expression, differentiation potential and support to angiogenesis *in vitro*.
The results obtained confirmed that these cells possess Vascular Stem Cell-like properties, due to the fact that they express MSC markers, display trilineage differentiation potential, differentiate towards vascular smooth muscle and endothelial cell phenotypes, and support angiogenesis *in vitro*.

In conclusion, the continuous involvement of the porcine animal model in the biomedical research, as the continuous advances achieved using this species in translational medicine, support the need for alternative methods to animal experimentation involving pigs.

Both the models described in the present manuscript were properly characterized and could be useful to the study of vascular biology. Moreover, both the models aim to reduce the use of experimental animals and to refine animal based-trials.

Because of the European Legislation and the Laboratory Animal Science guidelines there is no question of the relevance of further research concerning alternative methods to animal experimentation within the next future.
The present research aims to be a small, but significant, contribution to this important and necessary field of study.

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