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PROJECT FINAL REPORT

Grant Agreement number: 328695

Project acronym: GENE

Project title: Genomic screening of the Embryo for Novel targets in the tumor Endothelium

Funding Scheme: Marie Curie Intra-European Fellowships (IEF), FP7-PEOPLE-2012-IEF

Period covered: **from** 01/05/2013 **to** 30/04/2015

Name of the scientific representative of the project's co-ordinator[1], Title and Organisation:

Prof. Arjan W. Griffioen, Stichting VU-VUMC

Tel: +31204443374

Fax: +31204443844

E-mail: aw.griffioen@vumc.nl

Project website Error! Bookmark not defined. **address:** www.mcgene.eu

[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement.

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1 PUBLISHABLE SUMMARY

1.1 Executive summary

Angiogenesis is the formation of new blood vessels from pre-existing vasculature. In the healthy body angiogenesis takes place during wound healing or the growth of the endometrium. However, in diseases abnormal angiogenesis may occur. An example is the excess of angiogenesis in cancer, meaning that a tumor actively recruits blood vessels to supply itself with oxygen and nutrients.

Currently, there are several angiogenesis inhibitors on the market for treatment of cancer. Most of these drugs target pro-angiogenesis signaling by tumor cells through neutralization of growth factors or by inhibiting kinase activity of growth factor receptors. Although these drugs experience (commercial) success, it is now becoming evident that these agents, in general, only have moderate effect on survival rates of patients. Furthermore, these angiogenesis inhibitors show severe side effects in patients.

The aim of this project was to identify novel specific markers of angiogenic tumor endothelium, which we can target with specific drugs. Advantages are that this approach is independent of the growth factor production by tumor cells, which reduces the risk of tumor cell mutation into drug resistant variants. In addition, since the identified targets are specific to the tumor endothelial cells, toxic side effects will be minimized.

Our hypothesis was that certain genes with an exclusive function in the embryo also are present in the tumor vasculature. Therefore we have isolated the full transcriptome of mouse embryos at early and late developmental stages, and compared it with adult mouse isolates to identify the embryo-specific genes. Next we screened the transcriptome of tumor endothelial cells for the expression of the found embryo-specific genes. For the genomic screen next generation sequencing was used to maximize the identification of specific genes or splice variants. In our analysis we found 24 candidate genes, which are 100-fold higher expressed in tumor endothelial cells and embryo compared to the adult mouse. We validated the expression of the 10 most promising (extracellular expressed) candidate genes in the source material and continued with three of these targets. Currently, we are testing the targets in *in vitro* angiogenesis assays and are making vaccines against these targets. Future directions are to test the developed vaccines in preclinical mouse models and to produces monoclonal antibodies against the identified target molecules.

1.2 Project context and objectives

Sustained tumor growth and metastasis depend on the formation of new blood vessels. The process of neovascularization is also known as angiogenesis (Figure 1). Therefore, blocking of tumor angiogenesis is used as an opportunity for intervention in tumor growth (1). (Figure 1)

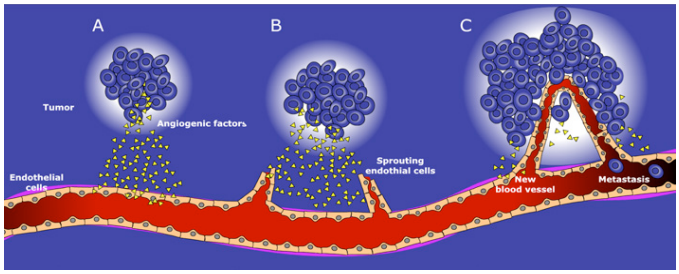

Figure 1. The angiogenesis cascade. **A.** A growing tumor produces angiogenic factors in order to attract blood vessels. **B.** Endothelial cells (EC) in nearby blood vessels start to migrate and grow towards the tumor. **C.** A new blood vessel is formed which provides the tumor with potential for further outgrowth as well as opportunities for metastasis formation.

Currently, several angiogenesis inhibitors are approved for the treatment of cancer and many others are in late-stage clinical testing. Most of these drugs target pro-angiogenesis signaling by tumor cells through neutralization of growth factors (e.g. anti-VEGF antibodies like bevacizumab/Avastin® from

Genentech/Roche), or by inhibiting kinase activity of the growth factor receptors (*e.g.* sunitinib/Sutent® from Pfizer). Although these compounds experience (commercial) success, it is now becoming evident that these agents, in general, only have a moderate effect on survival rates of patients. For example, bevacizumab prolongs survival in colorectal cancer patients on average by only 3-4 month (2). Part of this limited therapeutic effect is due to an induction of drug resistance that develops upon the inhibition of tumor-derived growth (3). Besides their limited activity, current angiogenesis inhibitors furthermore show severe side effects in patients (4, 5). In order to be able to improve anti-angiogenesis treatment, specific markers of the angiogenic tumor endothelium are required. This would allow intervention that is independent of the growth factor production by tumor cells, reducing the risk of tumor cell mutation into drug resistant variants. In addition if the identified targets were specific to tumor endothelial cells, toxic side effects would be minimized.

A critical step in targeting tumor vasculature is the identification of markers on tumor endothelial cells (EC). ECs in a particular microenvironment interact with numerous types of other cells and molecules. As a result, ECs in different tissues have specific functions, which is reflected in their gene expression profiles. It is this altered gene expression that may be exploited for therapeutic or diagnostic use. Several molecules have been found to be overexpressed in activated (tumor) endothelium. Among these molecules are β1-integrin (6), αvβ3-integrin (7), CD36 (8), CD44 (9) and plexin-D1 (10).

To date, only a very limited number of studies have attempted to characterize the gene expression profile of human tumor endothelium at a genome wide scale. Since only approximately 1% of the cells in a tissue are of endothelial origin, the major challenge in doing so is to obtain sufficiently pure populations of cells. The most commonly used method is to label ECs in single cell suspensions with antibodies and to subsequently capture those using magnetic beads. Four publications used serial analysis of gene expression (SAGE) to map gene expression of ECs isolated from colon, breast and malignant glioma (13-16). These studies identified targets but it was found that they were also overexpressed in physiologically activated endothelium rendering these targets rather unsuitable for therapeutic anti-cancer strategies.

In search for new fully specific targets in the tumor endothelium we hypothesized that certain genes with an exclusive function in the embryonic stage, also are expressed by the tumor vasculature. Therefore we decided to isolate the full transcriptome of mouse embryos at early and late developmental stages, and to compare it with the adult mouse transcriptome to be able to identify the embryo-specific genes. Next we screened the transcriptome of tumor endothelial cells for the expression of the found embryo-specific genes. For the genomic screen next generation sequencing was used to maximize the identification of specific genes or splice variants.

Therefore we have isolated the full transcriptome of mouse embryos at early and late developmental stages, and compared it with adult mouse isolates to identify the embryo-specific genes. Next we screened the transcriptome of tumor endothelial cells for the expression of the found embryo-specific genes. Currently, on going are the validation of a few targets in *in vitro* angiogenesis assays and the production of vaccines against these targets. The applicability of the developed vaccines for treatment of cancer will be tested in preclinical mouse tumor models. Finally, the human ortholog of the most promising gene candidates will be identified and validated for overexpression in human tumors. The ultimate long-term goal of our research line is to develop a new treatment approach for cancer.

Research Objectives

1. Identification of new fully specific markers of the mouse tumor endothelium by screening the embryonic transcriptome, to be used for anti-vascular, anti-cancer therapy.
2. Validation of the novel targets for overexpression and function in endothelial cells.
3. Development of drugs that specifically bind to the selected targets.
4. Testing of the novel drugs in *in vitro* and *in vivo* preclinical models.

5. Translation of the technology for treatment of patients, *i.e.* finding human orthologs.

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1.3 Main S&T results/foregrounds

The GENE project has achieved three out of five Scientific & Technological target objectives established at the start of the project.

As main result we identified 24 candidate genes, which are 100-fold higher expressed in tumor endothelial cells and embryo compared to the adult mouse, from our screen.

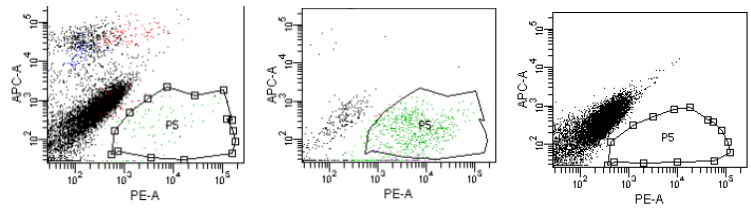
We validated the expression of the 10 most promising (extracellular expressed) candidate genes in the source material and continued with three of these targets in functional experiments and the development of vaccines.

With regards to the S&T objectives of the project, in line with the initial research objectives of the GENE project, the main results are indicated in the following.

1. Identification of new fully specific markers of the mouse tumor endothelium by screening the embryonic transcriptome, to be used for anti-vascular, anti-cancer therapy.

The full transcriptomes of two adult male C57BL/6 mice and mouse embryos from early (embryonic day 11) and late developmental (embryonic day 18) stages were isolated. We decided to use mouse embryos from embryonic day 11 (E11) instead of embryonic day 6 (E6) as stated in the research proposal, since isolation of E6 embryos is difficult due to their small size. Furthermore, organogenesis and thus angiogenesis (required for proper perfusion of the developing organs) takes place from E7 until E14 and we thus will be able to detect genes involved in angiogenesis in these embryonic stages.

We also isolated fresh endothelial cells from mouse B16-F10 melanoma tissue and normal mouse organs by high-speed fluorescence-activated cell sorting (FACS). For separation of the endothelial population we used the markers CD31/CD34 and the pan-leukocyte marker CD45, to be able to distinguish the endothelial cells from CD31/CD45^{+} macrophages (Figure 1 a-c). We used the Trizol isolation method to prepare total RNA (the full transcriptome) from all samples. A sufficient amount of total RNA and of good quality (RNA integrity number 7-8) could be isolated from all samples (Figure 1d). Next generation sequencing on the RNA isolates was performed on Illumina Hiseq 2000 equipment at the genomics core facility at the Netherlands Cancer Institute (NKI). The NKI also provided assistance with the bioinformatics analysis. In our analysis we found 24 candidate genes, which are a 100-fold ($p< 0.05$) higher expressed in tumor endothelial cells and the embryo compared to the adult mouse. We set the a cut-off value for the fold-expression at 100, because the amount of endothelial cells in the body represents about 1% of the whole body's cell population. After looking at the cellular localization of the targets we choose 10 genes to proceed with in validation experiments.



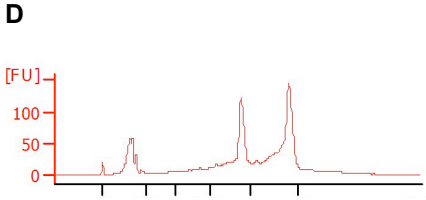


Figure 1. Representative FACS data and RNA quality plot. A) Cell sort of CD31/CD34 positive tumor endothelial cells, PE stained (gate P5). The APC channel indicates CD45 cells. B) Purity check of sorted population. C) Sort of unstained tumor suspension. D) Bioanalyzer plot of total RNA isolated from sorted tumor endothelial cells.

Completed deliverables

D1: Isolation of the full transcriptome of mouse embryos and adult mice.

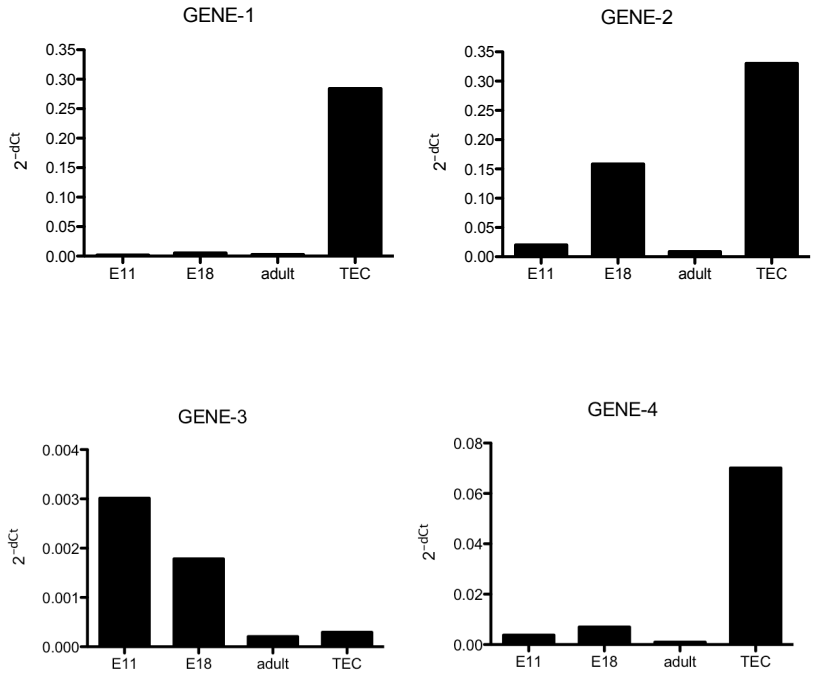
D2: Identification of embryo-specific genes.

D3: Isolation of tumor endothelial cells from mouse tumor material.

D4: Bioinformatics to find tumor endothelial cell genes present in the pool of embryonic genes.

2. Validation of the novel targets for overexpression and function in endothelial cells.

We have validated the expression of the 10 chosen target genes in the source material (the RNA isolates from embryo, adult mouse and tumor endothelium) by real time (RT)-PCR (Figure 2). The original names of the genes have been changed to GENE and have been given a number, since the obtained data are confidential.



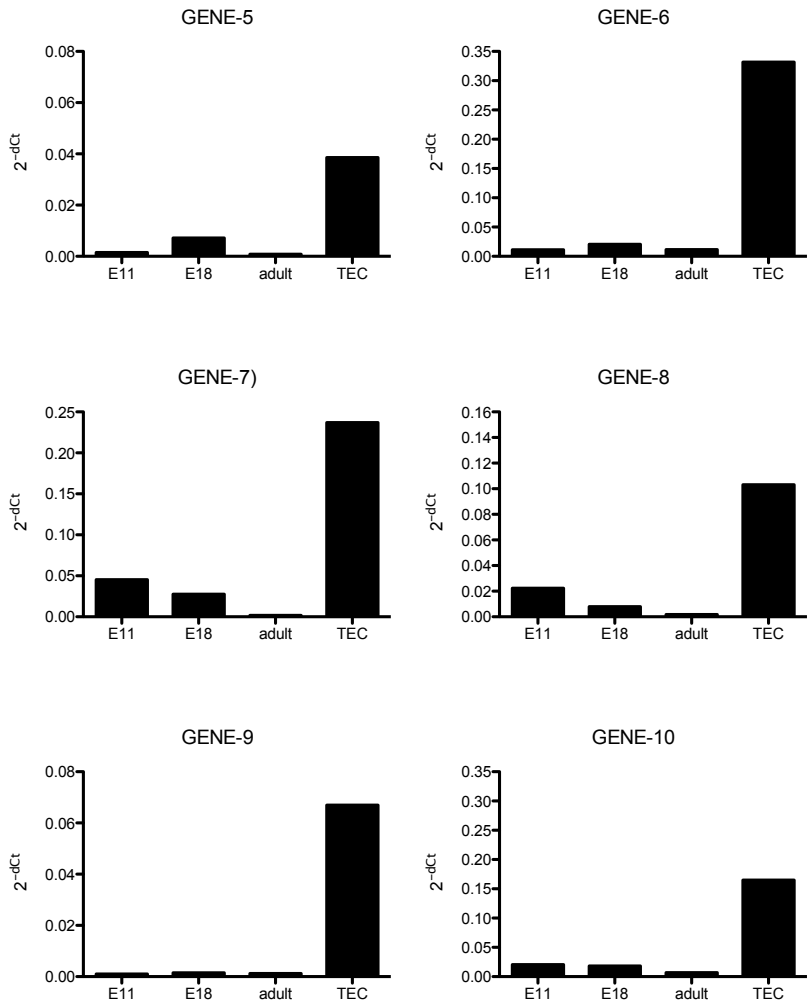


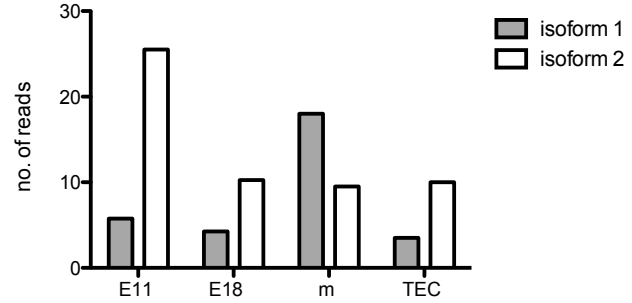
Figure 2. Validation of target gene expression in source material. The graphs show the expression at the mRNA level of the 10 different chosen target genes (named GENE-1 to GENE-10).

Before determining the expression in our source material we validated our primers, to be able to have a range of Ct values within which the expression can be considered reliable. We also used melting curves of the primers to be able to distinguish between primer dimer formation and amplicon/ amplification signal. Based on the obtained qPCR results and the RNA sequencing results we decided to continue with three out of 10 target genes (GENE-1, GENE-2 and GENE-4). We choose genes with a reasonable expression level to be able to detect any effect. Furthermore, we did an extensive literature search and found GENE-1 and GENE-4 to be involved in angiogenesis. GENE-2 was chosen because of its presence during embryonic development and in tumor endothelium. It has to be noted that the low expression/absence of most of the genes on embryonic day E11 or E18 not necessarily means that these genes are not switched on/present during embryonic development since we only analyzed two different embryonic stages. These genes thus still could be highly present on other embryonic days.

One of the reasons that RNA sequencing was performed in this project was to be able to identify protein splice variants. We have identified at least one interesting splice variant, which has an enhanced expression in tumor endothelium compared to normal endothelium. In Figure 3a the splicing pattern for the different isoforms of this gene are shown. We have validated the expression of these splice variants by real time RT-PCR and could confirm that the expression of isoform 2 is enhanced in

both mouse embryos (embryonic day E11 and E18) and in tumor endothelium (TEC) compared to normal healthy adult (m).

A



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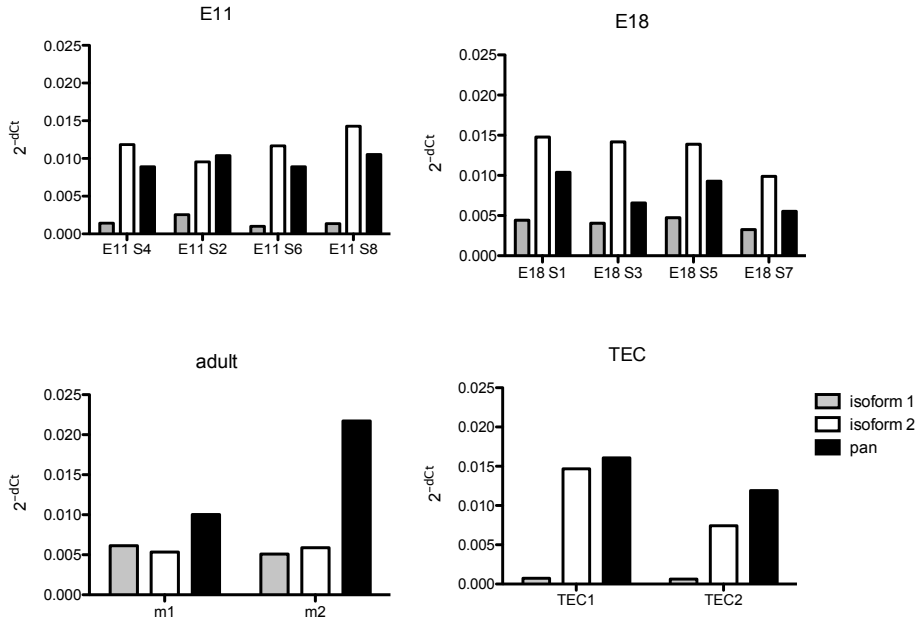

Figure 3. Specific splice variant expression in the embryo and tumor endothelium. A) Number of reads per isoform in the different samples. Embryos and TEC mainly express isoform 2 whereas in the healthy adult the isoform 1 is most frequent. B) Expression of the different isoforms on mRNA level. (embryonic day E11 (E11), embryonic day E18 (E18), mouse 1 (m1), mouse 2 (m2), tumor endothelium (TEC))

According to the work plan, work package 1 (WP1) – target identification and validation has been fully completed. The project deliverables D1-D4 have been accomplished.

3. Testing of the novel drugs in in vitro and in vivo preclinical models

We performed a literature search on the three selected target genes, to obtain knowledge about expression and function. For all the three chosen target genes we were able to identify human orthologs. Therefore we also designed primers for the human target sequences. To specifically study

the function of the selected genes in angiogenesis we started with siRNA knock-down (loss-of-function) experiments in human umbilical vein endothelial cells (HUVEC). The knock-down efficiency will be evaluated by qPCR on RNA will isolated from the siRNA-treated cells. Currently the knock-down experiments are ongoing.

We also tested if the ten identified target genes were specific to endothelial cells and thus not present in tumor cells. Therefore we isolated mRNA from B16-F10 melanoma cells (tumor cells) and Bend5 endothelial cell (mouse endothelial cell line). Indeed most of the ten target genes showed a very low expression in the B16-F10 tumor cells (Figure 4a). A higher expression level could be detected in Bend5 endothelial cells (Figure 4b) for some of the target genes. One might not expect expression of the target genes in normal endothelial cells. However, the Bend5 endothelial cell line is derived from immortalized mouse brain endothelial cells, which makes these cells more tumor endothelial cell-like.

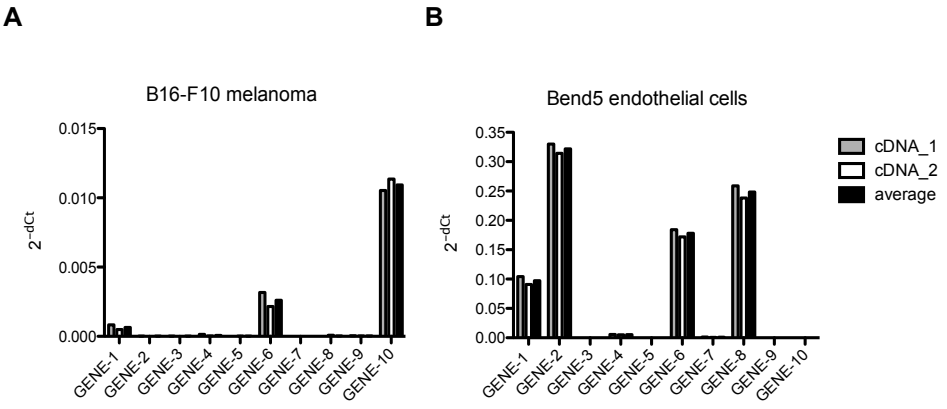

Figure 4. Expression of target genes in tumor cells and endothelial cells. A) mRNA levels of the 10 chosen target genes in B16-F10 melanoma cells. B) mRNA levels of the target genes in Bend5 mouse endothelial cells.

In addition we evaluated the expression of two of the chosen target genes in HUVEC and RF24 cells (immortalized human vascular endothelial cells) (Figure 5a and 5b). Both cell lines show a low to very low expression of the target genes, below a 2^{-dCt} value of 0.05, as compared to their expression in tumor endothelial cells. This indicates that cell culture of HUVEC might slightly induce the expression of these target genes, but that HUVEC largely retain their normal phenotype. Although RF24 are immortalized endothelial cells these cells barely express the two selected target genes (GENE-2 and GENE-4).

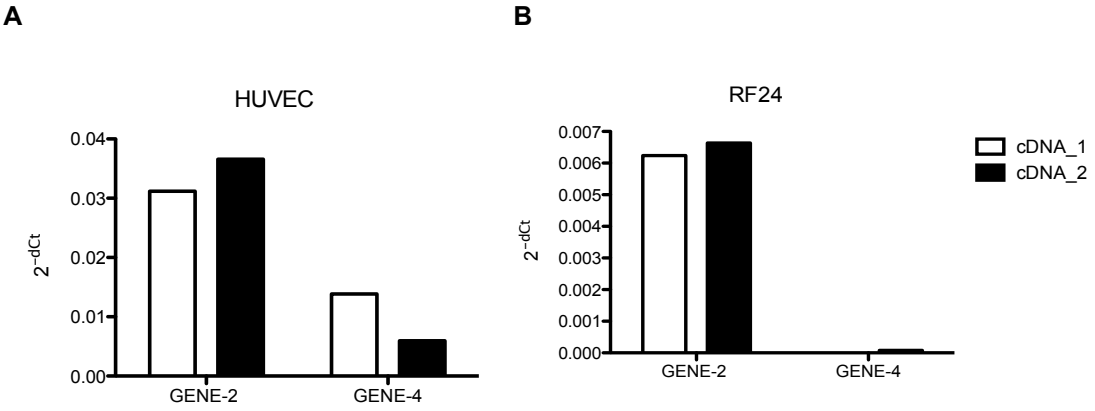

Figure 5. Expression of chosen target genes in human endothelial cell lines. A) mRNA level in HUVEC. B) mRNA level in RF24.

Currently, siRNA knock-down experiments are preformed in HUVEC for GENE-4 to study the effect of gene knock-down on migration, proliferation, tube formation, apoptosis and cell adhesion. For this purpose we choose HUVEC cells for the knock-down experiments, since these cells show the highest expression level of our target gene and it is expected that an effect of the knock-down can be easily observed.

For the selected target genes GENE-1 and GENE-2 commercial monoclonal antibodies are available. Both antibodies are able to detect the mouse and human form of the proteins. Immunohistochemistry was performed on mouse CT26 colon carcinoma tumour sections. Staining of native protein for both GENE-1 and GENE-2 could be detected in the tumour vasculature and tumour cells (Figure 6a, b). In addition, we stained human colon carcinoma tissue, normal colon and normal placenta for GENE-1 and GENE-2 and observed that expression of both genes is enhanced in tumour tissue (Figure 6).

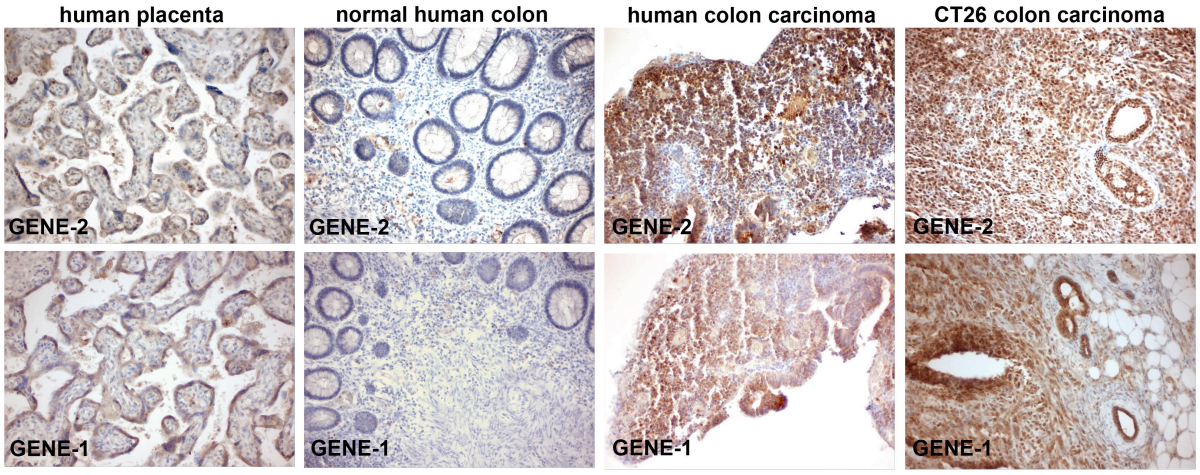

Figure 6. Staining of different tissues for GENE-1 and GENE-2. magnification 200x

The function of the selected target genes was addressed in the murine endothelial cell line SVEC. Migration ability, after addition of different concentrations of monoclonal antibody, was measured in a scratch assay. In this assay a wound is made and the size of the wound area is measured at time point zero and eight hours after scratching and addition of antibody. For both target genes migration was inhibited by 20-40% depending on the antibody concentration used (Figure 7a, b). As a positive control we used the tyrosine kinase inhibitor sunitinib, which is a potent inhibitor of the vascular endothelial growth factor receptor 2 (VEGFR2).

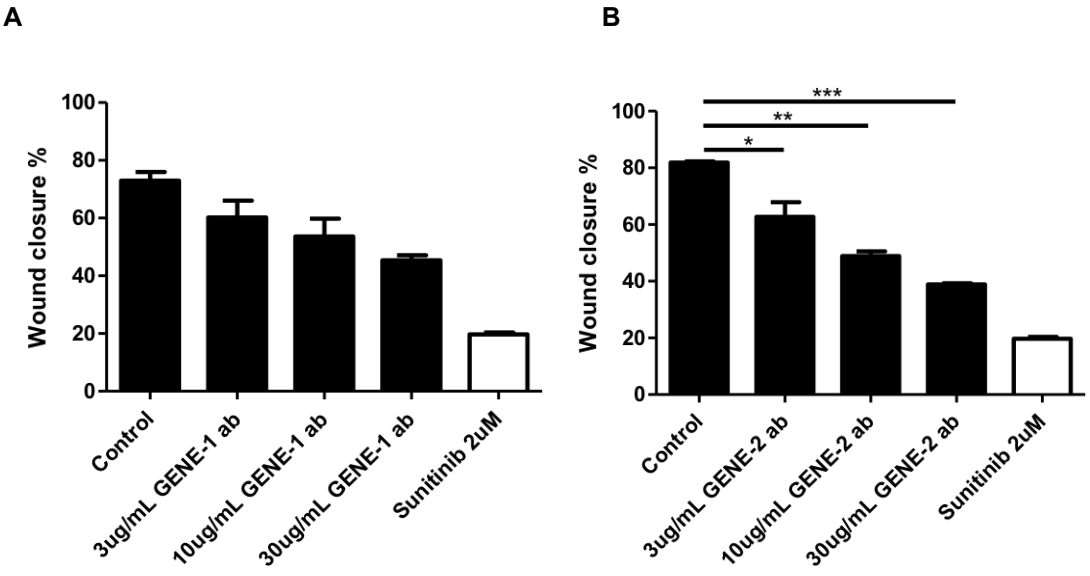

Figure 7. Migration is inhibited by addition of antibody against the target genes. In this assay wound closure is measured (%) and thus less wound closure indicates reduced migration. A) Antibody targeting GENE-1 was added to SVEC cells in different concentrations. B) Antibody targeting GENE-2 was added to SVEC cells in different concentrations. At time point zero the scratch was made, at time point eight the gap width was determined. The tyrosine kinase inhibitor sunitinib was used as a positive control. $p<0.05$

Proliferation capacity of SVEC cells treated with antibody against GENE-1 or GENE-2 was determined by CellTiter-Glo® luminescent cell viability assay. In this assay the number of viable cells is measured by the quantification of ATP in which the luminescent signal is proportional to the amount of ATP present. Cells were treated for 48h with different concentrations of antibody and the signal was read out. No significant inhibition of proliferation by either of the antibodies added was observed in this assay (Figure 8 a, b).

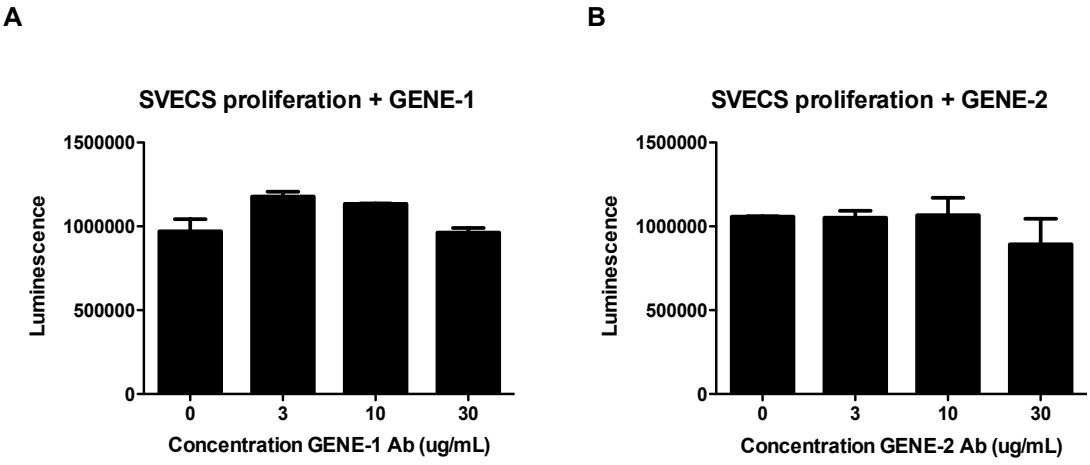

Figure 8. Cell proliferation is not inhibited by antibody against the target genes. In this assay cell viability was measured with CellTiter-Glo®. A) Antibody targeting GENE-1 is was added to SVEC cells in different concentrations. B) Antibody targeting GENE-2 is was added to SVEC cells in different concentrations.

The target gene GENE-2 is secreted by tumour endothelial cells, therefore we measured the secretion of this gene into the cell medium of SVEC cells. GENE-2 was detected by in enzyme linked immunosorbent assay (ELISA) in which different dilutions of the detection antibody (monoclonal antibody against GENE-2) were used. In Figure 9 it can be seen that GENE-2 was indeed secreted

into the cell medium of SVEC cells 24h after seeding of the cells and that the amount present in the medium is proportional to the cell number.

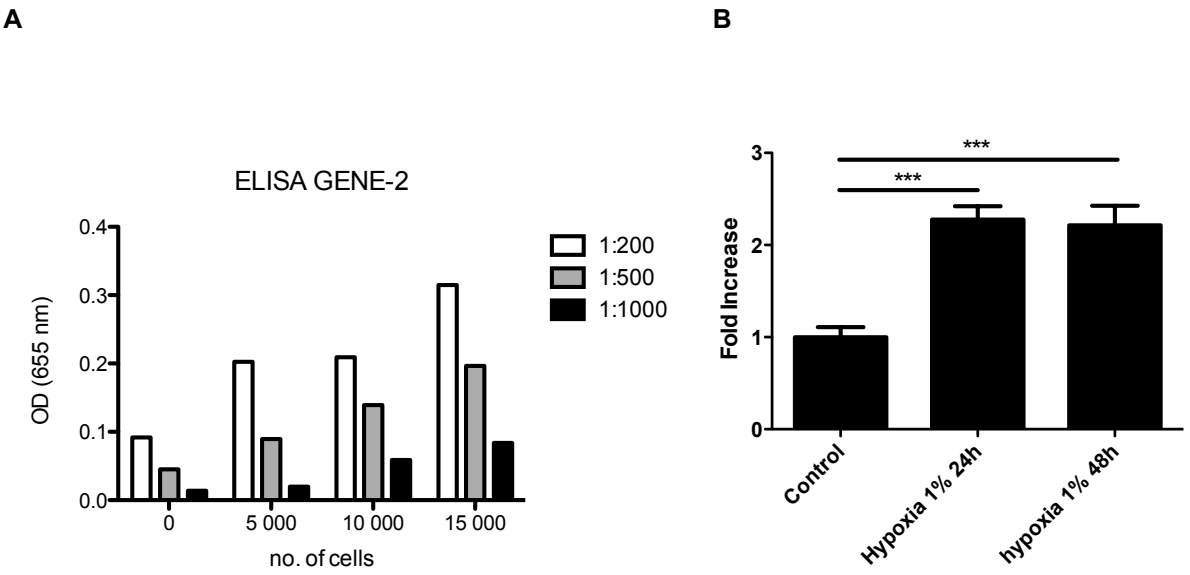

Figure 9. Secretion of GENE-2 into the cell culture medium is enhanced under hypoxia. The secretion of the protein GENE-2 into the cell culture medium was measured by indirect ELISA. A) Antibody dilutions used are 1:200, 1:500 and 1:1000. B) Secretion of GENE-2 into the culture medium is increased under hypoxia. $p<0.0001$

In a tumour there are hypoxic regions present and it is known that hypoxia can stimulate angiogenesis. Therefore we addressed if hypoxia could enhance the secretion of GENE-2 into the culture medium of SVEC cells. Cells were placed under hypoxia (1% oxygen) for 24h or 48h. We observed that under hypoxic culture conditions the secretion of GENE-2 was 2.5 fold increased compared to normoxic conditions (Figure 9b). In addition the inhibitory effect of the GENE-2 blocking antibody was greater under hypoxic conditions (Figure 10b) as compared to normoxic culture conditions (Figure 7b). Targeting GENE-2 was as effective as inhibition of the VEGFR2 by the anti-angiogenic drug sunitinib (Figure 10b), indicating that blocking GENE-2 might inhibit tumour angiogenesis *in vivo*. Similar to the effect observed for GENE-2 migration of SVEC under hypoxia was significantly inhibited by addition of an antibody against GENE-1 to the culture medium (Figure 10a). Again the inhibitory effect of the blocking antibody was greater under hypoxic compared to normoxic culture conditions (Figure 7a).

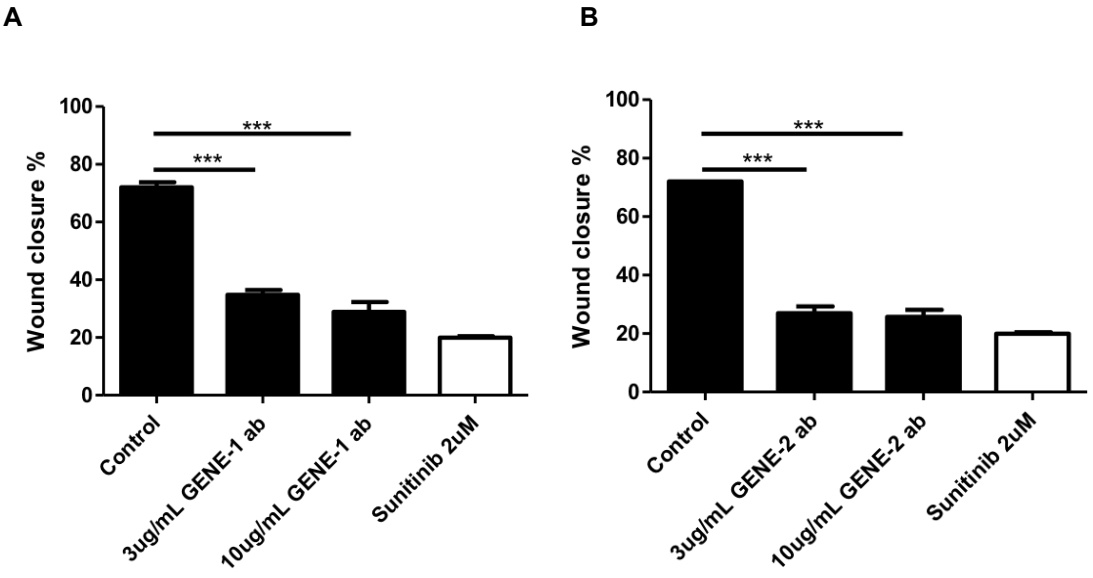

Figure 10. The effect of the antibodies on migration is greater under hypoxic culture conditions. In this assay wound closure is measured (%) and thus less wound closure indicates reduced migration. A) Antibody targeting GENE-1 was added to SVEC cells in different concentrations. B) Antibody targeting GENE-2 was added to SVEC cells in different concentrations. At time point zero the scratch was made, at time point eight the gap width was determined. The tyrosine kinase inhibitor sunitinib was used as a positive control. $p<0.0001$

We also studied if the different antibodies against the chosen target genes could inhibit proliferation under hypoxic condition, but could not observe any effect of the antibodies on proliferation (Figure 11a). However, similar to the effect observed for GENE-2 migration of SVEC under hypoxia was significantly inhibited by addition of an antibody against GENE-1 to the culture medium (Figure 11b). Again the inhibitory effect of the blocking antibody was greater under hypoxic compared to normoxic culture conditions (Figure 7a).

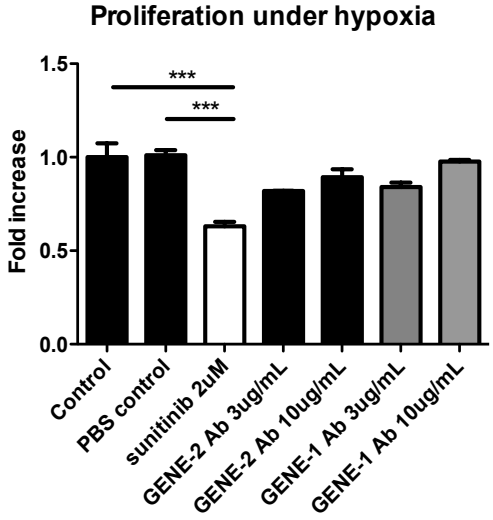

Figure 11. Proliferation under hypoxia is not blocked by an antibody against one of the target genes. A) Only the tyrosine kinase inhibitor sunitinib significantly blocks proliferation of SVEC under hypoxic conditions. $p<0.0001$

We are currently developing fusion proteins against the target genes GENE-1 and GENE-2 for *in vivo* preclinical vaccination tests in mice (as described in Huijbers *et al*., FASEB J 2010). The fusion proteins consist of a foreign (non-self) part, which is of bacterial origin, and a self-part, the to be targeted protein (GENE-1 or GENE-2). The fusion protein will be injected together with a potent adjuvant to induce an immune response against the self-antigen and inhibition of B16-F10 melanoma tumour growth will be measured. In Figure 12 the in *Escherichia coli* (*E. coli*) produced fusion proteins on SDS-PAGE gel stained with coomassie brilliant blue are shown. Both fusion proteins are fairly pure and it is anticipated that vaccination of mice can soon be initiated.

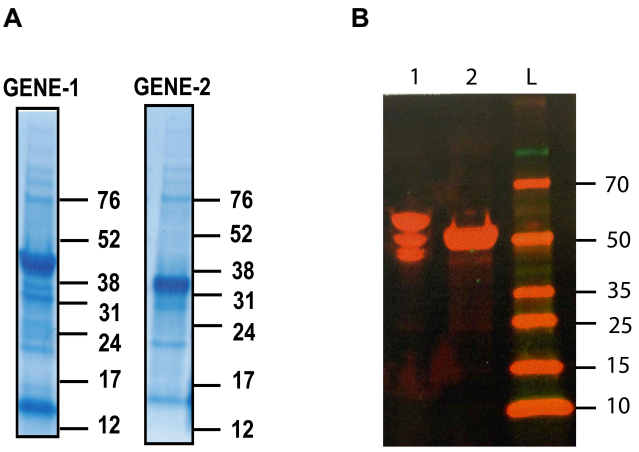

Figure 12. Fusion proteins on a reducing SDS-PAGE gel and western blot. A) In *E. coli* produced fusion proteins of GENE-1 (50 kDa) and GENE-2 (37 kDa). B) Western blot of TRX-vimentin fusion protein (lane 1, 67 kDa), vimentin (lane 2, 54 kDa), protein ladder (L). Mouse vimentin was detected with the anti-vimentin antibody RV202 (Santa Cruz).

In a previous screen we have identified the target gene vimentin as a tumor vascular marker (van Beijnum J, Blood 2006). For this target gene already many *in vitro* functional studies have been performed previously. Therefore we decided to develop a vaccine against vimentin. We have produced a fusion protein (TRX-vimentin) comprised of the self-part mouse vimentin and a foreign bacterial part thioredoxin (TRX) (Figure 12b). We also produced the mouse vimentin alone to be able to detect antibodies against vimentin in ELISA. Identity of the proteins was confirmed by western blot with an anti-vimentin antibody (Figure 12b).

We have vaccinated immunocompetent wild type mice (C57BL/6, BALB/c) four times with the fusion protein together with Freund's adjuvant (Figure 13). During the course of the experiment blood samples are taken to measure anti-vimentin antibodies by ELISA. When antibodies against vimentin are present in the sera of vaccinated mice tumor cells are injected subcutaneously in the flank of the mouse and tumor growth is monitored. We used two different subcutaneous tumor models B16-F10 melanoma and CT26 colon carcinoma.

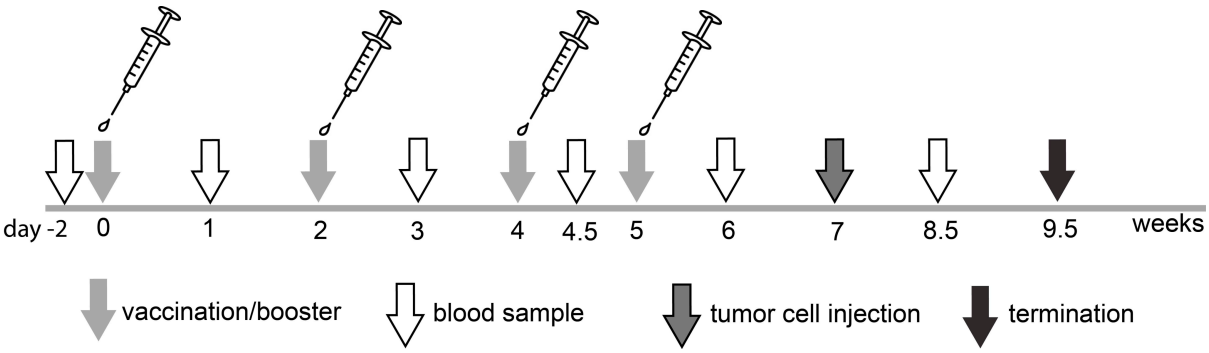

Figure 13. Experimental set-up vimentin vaccination study. Mice are vaccinated four times with fusion protein and adjuvant. Blood samples are taken to address anti-vimentin antibody levels by ELISA. In week 7 tumor cells are injected subcutaneously in the flank of the mouse and tumor growth is measured.

All mice responded well to vaccination and no side effects of the vaccine could be observed. Self-tolerance against the self-antigen was broken, since antibodies against vimentin were induced in the mice (Figure 14 a, c). In addition in both models, tumor growth was significantly inhibited by about 50% in the mice with anti-vimentin antibodies present in their sera (Figure 14 b,c).

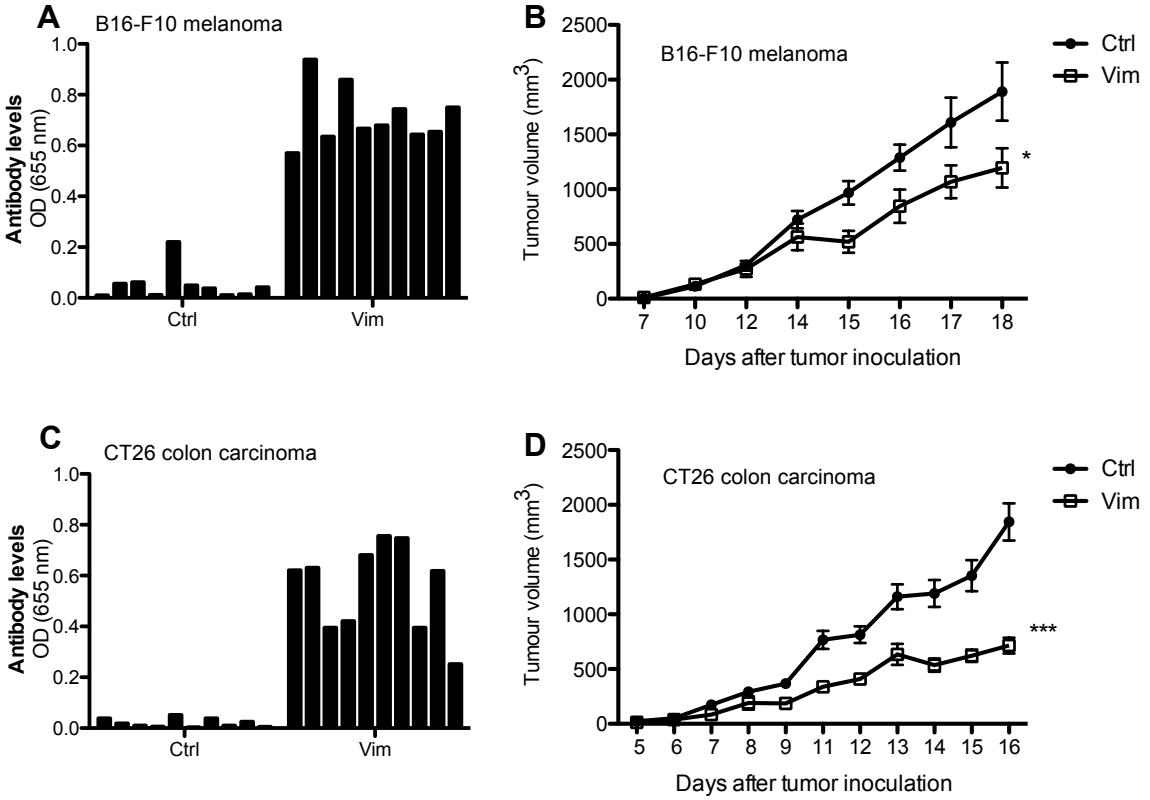

Figure 14. Vaccination against vimentin inhibits tumor growth in two different mouse models. A) Antibody levels in C57BL/6 mice one week prior to tumor cell inoculation (week 6). B) Tumor growth curves of B16-F10 melanoma. C) Antibody levels in BALB/c mice one week prior to tumor cell inoculation (week 6). D) Tumor growth curves of CT26 colon carcinoma. Control vaccinated mice (Ctrl), TRX-Vim vaccinated mice (Vim). p=0.0377 (B16-F10 model), p<0.0001 (CT26 model)

Preliminary data of the B16-F10 tumor model show that the number of blood vessels is significantly reduced in the TRX-vimentin treated tumors (Figure 15).

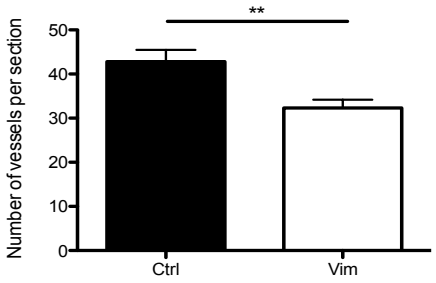

Figure 15. The number of tumor blood vessels is reduced after vaccination against vimentin. The number of blood vessels was counted per tumor section in control (Ctrl) and vimentin (Vim) treated tumors. p=0.0024

We also isolated the spleens of the vimentin vaccinated BALB/c mice for the production of monoclonal antibodies. The hybridoma and monoclonal antibody production is currently outsourced to the biotech company Eurogentec (Maastricht, The Netherlands).

Partially completed deliverables

D5: Different *in vitro* angiogenesis assays on mouse EC and primary EC

D6: Gain- and loss-of-function studies

D8: Antibody production

D11: Initial preclinical testing

D12: Publish results and present results at international meetings.

Work package 2 – target function and work package 3 - drug development have been partially completed.

4. Translation of the technology for treatment of patients, i.e. finding human orthologs.

Human orthologs have been identified for the 10 chosen target genes. This is very promising, which means that the technology can be transferred to humans and might eventually we used for treatment of patients with cancer.

5. Future directions

Fusion proteins for GENE-1 and GENE-2 are produced and recently vaccination studies in mice were started. Vaccination of mice is required for the production of monoclonal antibodies against the chosen target molecules, therefore no antibodies against the novel targets have been produced yet. The developed antibodies will be tested in mouse tumor models using a therapeutic set up, where treatment is started when established tumors of 10-20 mm^3 are present. In addition the developed vaccines will be tested for inhibition of tumor growth in mice. If antibody treatment and/or vaccination is successful antibodies and vaccines against the human ortholog of the target genes will be developed.

We have already initiated to patent the described method of target identification.

Since treatment of mice with the vimentin vaccine was successful we have initiated a collaboration with the Faculty of Veterinary Medicine at Utrecht University to perform a phase I clinical study in dogs. For this study dogs with prostate cancer will be recruited to test the vaccine in a therapeutic setting.

Uncompleted deliverables – work package 3 – drug development

D9: Confirmation and characterization of target interaction of the produced antibodies

D10: Search for endogenous binding molecules to the identified targets

1.4 Use and dissemination of foreground

To spread awareness of the research project a project website was built. The **GENE website** (http://www.mcgene.eu), through which general information on GENE is made available, played a major role in the dissemination of the project activities and results. The website was launched in June 2014 and was continuously populated with content during the lifetime of the project. The public results of the project are available on the website including a project description and peer-reviewed publications.

Peer-reviewed publications play an important role in the dissemination of the GENE results. Currently, a manuscript including the results of the embryonic screen is in progress and several other publications are expected to derive from this project in the near future.

The results of the GENE project have been presented at the 5th International Meeting on Angiogenesis, organized in March 2014 in Amsterdam. The abstract has been published in Angiogenesis (vol 17, no.1, January 2014).

To raise public awareness the EU Marie Curie fellow has participated twice in the TCS Amsterdam Marathon during the course of the project. In the year 2014 she was part of the fundraising website of the VUmc Cancer Center Amsterdam (http://www.helpdeheld.nl/). In addition, the high-quality Amsterdam-based newspaper 'Het Parool' interviewed her. In this interview the fellow was able to explain the research and the Marie Curie program.

In October 2014 the fellow hosted a group of nurses from Bergen (Norway) in the laboratory to whom she presented her work.

Once the data of the embryonic screen are published in a peer-reviewed journal a newspaper article for a national newspaper will be written to further raise public awareness. The project coordinator Prof. Dr. Griffioen is the owner of the spin-off company Angiotarget, through which we will look for investors to translate the developed treatment into the clinic. We also have interaction with the private sector through the academic technology transfer office (TTO) at the VU University. The TTO is currently assisting us with a patent application that is based on the results of the current project.

Further dissemination will take place by publication in scientific peer-reviewed journals. The following manuscripts are in preparation:

- Genomic screen of the embryo for novel targets in the tumor endothelium
- Vimentin vaccination paper
- Alternatives to bacterial thioredoxin to optimize the immune response against self-antigens
- Resistance mechanisms to anti-angiogenic therapy, Drug Resistance Updates, *to be submitted soon*

Section A (public)

A1: LIST OF SCIENTIFIC (PEER REVIEWED) PUBLICATIONS, STARTING WITH THE MOST IMPORTANT ONES

NO.	Title	Main author	Title of the periodical or the series	Number, date or frequency	Publisher	Place of publication	Year of publication	Relevant pages	Permanent identifiers[2] (if available)	Is/Will open access[3] provided to this publication?
1	*The great escape; hallmarks of resistance to anti-angiogenic therapy.*	*Van Beijnum JR (equal contribution)*	*Pharmacological Reviews*	*No 67, April 2015*	*Williams & Wilkins*	*Baltimore, US*	*2015*	*pp. 441 - 461*	*http://www.ncbi.nlm.nih.gov/pubmed/25769965*	*no*
2	*Vaccination approach to anti-angiogenic treatment of cancer.*	*Wentink MQ*	*Biochimica et Biophysica acta*	*No 1855, April 2015*	*Elsevier Pub. Co.*	*Amsterdam, The Netherlands*	*2015*	*pp. 155-171*	*http://www.ncbi.nlm.nih.gov/pubmed/25641676*	*no*
3	*Therapeutic vaccination against fibronectin ED-A attenuates progression of metastatic breast cancer*	*Femel J*	*Oncotarget*	*No 5, December 2014*	*Albany, N.Y.: Impact Journals*	*US*	*2014*	*pp. 12418-12427*	*http://www.impactjournals.com/oncotarget/index.php?journal=oncotarget&page=article&op=view&path[]=2628*	*yes*
4	*Vaccines targeting self-antigens: mechanisms and efficiency-determining parameters.*	*Saupe F (shared first authorship)*	*FASEB J*	*April 2015*	*Bethesda, Md.] : The Federation*	*US*	*2015*	*pp fj.15-271502. [Epub ahead of print]*	*http://www.ncbi.nlm.nih.gov/pubmed/25868727*	*no*
5	*CD44 enhances tumor aggressiveness by promoting tumor cell plasticity*	*Paulis YWJ (shared first authorship)*	*Oncotarget*	*April 2015*	*Albany, N.Y.: Impact Journals*	*US*	*2015*		*http://www.impactjournals.com/oncotarget/index.php?journal=oncotarget&page=article&op=view&path%5B%5D=3839*	*yes*

A2: LIST OF DISSEMINATION ACTIVITIES

NO.	Type of activities[4]	Main leader	Title	Date/Period	Place	Type of audience[5]	Size of audience	Countries addressed
1	*Conference*		*5th International Meeting on Angiogenesis*	*12-14 March 2014*	*Amsterdam*	*Scientific Community*	*250 participants*	*International*
2	*www.mcgene.eu*	*Huijbers E*	*GENE project website*	*June 2014- current*	*Amsterdam*	*Civil Society*		*International*
3	*TCS Amsterdam marathon*	*Huijbers E*		*October 2013*	*Amsterdam*	*Civil Society*		*Netherlands*
3	*TCS Amsterdam marathon*	*Huijbers E*		*October 2014*	*Amsterdam*	*Civil Society*		*Netherlands*
4	*Newspaper 'Het Parool' interview*	*Huijbers E*	*Amsterdammer van de dag*	*17 October 2014*	*Amsterdam*	Civil Society		*Netherlands*

[4] A drop down list allows choosing the dissemination activity: publications, conferences, workshops, web, press releases, flyers, articles published in the popular press, videos, media briefings, presentations, exhibitions, thesis, interviews, films, TV clips, posters, Other.

[5] A drop down list allows choosing the type of public: Scientific Community (higher education, Research), Industry, Civil Society, Policy makers, Medias, Other ('multiple choices' is possible).

Section B (Confidential[6] or public: confidential information to be marked clearly)

Part B1

B1: LIST OF APPLICATIONS FOR PATENTS, TRADEMARKS, REGISTERED DESIGNS, ETC.					
Type of IP Rights[7]:	Confidential Click on YES/NO	Foreseen embargo date dd/mm/yyyy	Application reference(s) (e.g. EP123456)	Subject or title of application	Applicant (s) (as on the application)
Patent	Yes	31-12-2015		*Embryonic screening method for target identification*	Prof. Dr. A.W. Griffioen, Dr. E.J.M. Huijbers

[6] Note to be confused with the "EU CONFIDENTIAL" classification for some security research projects.

[7] A drop down list allows choosing the type of IP rights: Patents, Trademarks, Registered designs, Utility models, Others.

Part B2

Type of Exploitable Foreground[8]	Description of exploitable foreground	Confidential Click on YES/NO	Foreseen embargo date dd/mm/yyyy	Exploitable product(s) or measure(s)	Sector(s) of application[9]	Timetable, commercial or any other use	Patents or other IPR exploitation (licences)	Owner & Other Beneficiary(s) involved
Commercial exploitation of R&D results	*Embryonic screening method for target identification*	*Yes*	*31/12/2015*	*Identification method, Vaccination strategy*	*1. Medical 2. Industrial*	*10 years*	*A patent is planned for 2015*	*Prof. Dr. A.W. Griffioen, Dr. E.J.M. Huijbers*

Explanation of the exploitable foreground, in particular:

The purpose of this work is to provide a screening method for the identification of novel specific target genes in the tumor endothelium. As a start we have screened only one tumor endothelial cell type for the expression of embryonic genes. Therefore this screening method can be extended to endothelial cells of other tumor types. Identified target genes with most potential for development of anti-cancer therapy will be patented in conjunction with the vaccination strategy. The technology transfer office of VUMC will take care of all intellectual property issues and will assist with the patent application. In addition the spin-off company of the host supervisor Angiotarget B.V. will license the developed technology.

We will test several of the identified targets preclinically in the vaccination strategy. The most promising target genes will be used for further development of a human anti-cancer vaccine. In order to start a phase I clinical trial with the developed vaccine, the vaccine has to be produced under GMP and a potent adjuvant able to break immune tolerance in humans has to be available. Furthermore toxicity studies in monkeys will be performed to foresee possible side effects of the vaccine in humans.

It is expected that within the time frame of 10 years a vaccine against one of the identified targets can be used for treatment of cancer. Scientific results will be published in high-ranked international peer-reviewed journals.

[19] A drop down list allows choosing the type of foreground: General advancement of knowledge, Commercial exploitation of R&D results, Exploitation of R&D results via standards, exploitation of results through EU policies, exploitation of results through (social) innovation.
[9] A drop down list allows choosing the type sector (NACE nomenclature) : http://ec.europa.eu/competition/mergers/cases/index/nace_all.html

2 REPORT ON SOCIETAL IMPLICATIONS

Replies to the following questions will assist the Commission to obtain statistics and indicators on societal and socio-economic issues addressed by projects. The questions are arranged in a number of key themes. As well as producing certain statistics, the replies will also help identify those projects that have shown a real engagement with wider societal issues, and thereby identify interesting approaches to these issues and best practices. The replies for individual projects will not be made public.

A General Information *(completed automatically when **Grant Agreement number** is entered.*

Grant Agreement Number:	328695
Title of Project:	Genomic screening of the Embryo for Novel targets in the tumor Endothelium
Name and Title of Coordinator:	Prof. Arjan Griffioen

B Ethics

1. Did your project undergo an Ethics Review (and/or Screening)? • If Yes: have you described the progress of compliance with the relevant Ethics Review/Screening Requirements in the frame of the periodic/final project reports? Special Reminder: the progress of compliance with the Ethics Review/Screening Requirements should be described in the Period/Final Project Reports under the Section 3.2.2 *'Work Progress and Achievements'*	***No***
2. Please indicate whether your project involved any of the following issues (tick box) :	***YES***
RESEARCH ON HUMANS	
• Did the project involve children?	
• Did the project involve patients?	
• Did the project involve persons not able to give consent?	
• Did the project involve adult healthy volunteers?	
• Did the project involve Human genetic material?	
• Did the project involve Human biological samples?	
• Did the project involve Human data collection?	
RESEARCH ON HUMAN EMBRYO/FOETUS	
• Did the project involve Human Embryos?	
• Did the project involve Human Foetal Tissue / Cells?	
• Did the project involve Human Embryonic Stem Cells (hESCs)?	
• Did the project on human Embryonic Stem Cells involve cells in culture?	
• Did the project on human Embryonic Stem Cells involve the derivation of cells from Embryos?	
PRIVACY	
• Did the project involve processing of genetic information or personal data (eg. health, sexual lifestyle, ethnicity, political opinion, religious or philosophical conviction)?	
• Did the project involve tracking the location or observation of people?	
RESEARCH ON ANIMALS	
• Did the project involve research on animals?	x
• Were those animals transgenic small laboratory animals?	
• Were those animals transgenic farm animals?	

• Were those animals cloned farm animals?	
• Were those animals non-human primates?	
RESEARCH INVOLVING DEVELOPING COUNTRIES	
• Did the project involve the use of local resources (genetic, animal, plant etc)?	
• Was the project of benefit to local community (capacity building, access to healthcare, education etc)?	
DUAL USE	
• Research having direct military use	No
• Research having the potential for terrorist abuse	No

C Workforce Statistics

3. Workforce statistics for the project: Please indicate in the table below the number of people who worked on the project (on a headcount basis).

Type of Position	Number of Women	Number of Men
Scientific Coordinator		
Work package leaders		
Experienced researchers (i.e. PhD holders)	1	
PhD Students		
Other		

4. How many additional researchers (in companies and universities) were recruited specifically for this project?	
Of which, indicate the number of men:	none

D Gender Aspects

5.	**Did you carry out specific Gender Equality Actions under the project**?	○ x	Yes No

6. Which of the following actions did you carry out and how effective were they?

		Not at all effective				Very effective
❑	Design and implement an equal opportunity policy	○	○	○	○	○
❑	Set targets to achieve a gender balance in the workforce	○	○	○	○	○
❑	Organise conferences and workshops on gender	○	○	○	○	○
❑	Actions to improve work-life balance	○	○	○	○	○
x	Other:	Not applicable				

7. Was there a gender dimension associated with the research content – i.e. wherever people were the focus of the research as, for example, consumers, users, patients or in trials, was the issue of gender considered and addressed?

○ Yes- please specify

x No

E Synergies with Science Education

8. Did your project involve working with students and/or school pupils (e.g. open days, participation in science festivals and events, prizes/competitions or joint projects)?

○ Yes- please specify

x No

9. Did the project generate any science education material (e.g. kits, websites, explanatory booklets, DVDs)?

○ Yes- please specify

x No

F Interdisciplinarity

10. Which disciplines (see list below) are involved in your project?

x Main discipline[1]: 3.1 Basic medicine

x Associated discipline[1]: 1.5 Biological sciences

○ Associated discipline[1]:

G Engaging with Civil society and policy makers

11a	**Did your project engage with societal actors beyond the research community?** *(if 'No', go to Question 14)*	○ x	Yes No

11b If yes, did you engage with citizens (citizens' panels / juries) or organised civil society (NGOs, patients' groups etc.)?

○ No

○ Yes- in determining what research should be performed

○ Yes - in implementing the research

○ Yes, in communicating /disseminating / using the results of the project

[1] Insert number from list below (Frascati Manual).

11c In doing so, did your project involve actors whose role is mainly to organise the dialogue with citizens and organised civil society (e.g. professional mediator; communication company, science museums)?	○ ○	Yes No

12. Did you engage with government / public bodies or policy makers (including international organisations)

- ○ No
- ○ Yes- in framing the research agenda
- ○ Yes - in implementing the research agenda
- ○ Yes, in communicating /disseminating / using the results of the project

13a Will the project generate outputs (expertise or scientific advice) which could be used by policy makers?

- ○ Yes – as a **primary** objective (please indicate areas below- multiple answers possible)
- ○ Yes – as a **secondary** objective (please indicate areas below - multiple answer possible)
- ○ No

13b If Yes, in which fields?

Agriculture		Energy		Human rights	
Audiovisual and Media		Enlargement		Information Society	
Budget		Enterprise		Institutional affairs	
Competition		Environment		Internal Market	
Consumers		External Relations		Justice, freedom and security	
Culture		External Trade		Public Health	
Customs		Fisheries and Maritime Affairs		Regional Policy	
Development Economic and Monetary Affairs		Food Safety		Research and Innovation	
		Foreign and Security Policy		Space	
Education, Training, Youth		Fraud		Taxation	
Employment and Social Affairs		Humanitarian aid		Transport	

13c If Yes, at which level?

- ○ Local / regional levels
- ○ National level
- ○ European level
- ○ International level

H Use and dissemination

14. How many Articles were published/accepted for publication in peer-reviewed journals?	**5**
To how many of these is open access[2] provided?	**2**
How many of these are published in open access journals?	**2**
How many of these are published in open repositories?	**0**
To how many of these is open access not provided?	**3**
Please check all applicable reasons for not providing open access:	
❑ publisher's licensing agreement would not permit publishing in a repository ❑ no suitable repository available ❑ no suitable open access journal available x no funds available to publish in an open access journal ❑ lack of time and resources ❑ lack of information on open access ❑ other[3]: ……	

15. How many new patent applications ('priority filings') have been made? *("Technologically unique": multiple applications for the same invention in different jurisdictions should be counted as just one application of grant).*		**1**
16. Indicate how many of the following Intellectual Property Rights were applied for (give number in each box).	Trademark	
	Registered design	
	Other	
17. How many spin-off companies were created / are planned as a direct result of the project?		**none**
Indicate the approximate number of additional jobs in these companies:		

18. Please indicate whether your project has a potential impact on employment, in comparison with the situation before your project:

❑	Increase in employment, or	❑	In small & medium-sized enterprises
❑	Safeguard employment, or	❑	In large companies
❑	Decrease in employment,	x	None of the above / not relevant to the project
❑	Difficult to estimate / not possible to quantify		

19. For your project partnership please estimate the employment effect resulting directly from your participation in Full Time Equivalent (*FTE = one person working fulltime for a year*) jobs:	*Indicate figure:*

[2] Open Access is defined as free of charge access for anyone via Internet.
[3] For instance: classification for security project.

Difficult to estimate / not possible to quantify	❑

I Media and Communication to the general public

20. As part of the project, were any of the beneficiaries professionals in communication or media relations?

○ Yes x No

21. As part of the project, have any beneficiaries received professional media / communication training / advice to improve communication with the general public?

○ Yes x No

22 Which of the following have been used to communicate information about your project to the general public, or have resulted from your project?

x	Press Release	❑	Coverage in specialist press
❑	Media briefing	❑	Coverage in general (non-specialist) press
❑	TV coverage / report	❑	Coverage in national press
❑	Radio coverage / report	❑	Coverage in international press
❑	Brochures /posters / flyers	x	Website for the general public / internet
❑	DVD /Film /Multimedia	x	Event targeting general public (festival, conference, exhibition, science café)

23 In which languages are the information products for the general public produced?

x	Language of the coordinator	x	English
❑	Other language(s)		

***Question F-10*:** Classification of Scientific Disciplines according to the Frascati Manual 2002 (Proposed Standard Practice for Surveys on Research and Experimental Development, OECD 2002):

FIELDS OF SCIENCE AND TECHNOLOGY

1. NATURAL SCIENCES

1.1 Mathematics and computer sciences [mathematics and other allied fields: computer sciences and other allied subjects (software development only; hardware development should be classified in the engineering fields)]

1.2 Physical sciences (astronomy and space sciences, physics and other allied subjects)

1.3 Chemical sciences (chemistry, other allied subjects)

1.4 Earth and related environmental sciences (geology, geophysics, mineralogy, physical geography and other geosciences, meteorology and other atmospheric sciences including climatic research, oceanography, vulcanology, palaeoecology, other allied sciences)

1.5 Biological sciences (biology, botany, bacteriology, microbiology, zoology, entomology, genetics, biochemistry, biophysics, other allied sciences, excluding clinical and veterinary sciences)

2 ENGINEERING AND TECHNOLOGY

2.1 Civil engineering (architecture engineering, building science and engineering, construction engineering, municipal and structural engineering and other allied subjects)

2.2 Electrical engineering, electronics [electrical engineering, electronics, communication engineering and systems, computer engineering (hardware only) and other allied subjects]

2.3. Other engineering sciences (such as chemical, aeronautical and space, mechanical, metallurgical and materials engineering, and their specialised subdivisions; forest products; applied sciences such as

geodesy, industrial chemistry, etc.; the science and technology of food production; specialised technologies of interdisciplinary fields, e.g. systems analysis, metallurgy, mining, textile technology and other applied subjects)

3. MEDICAL SCIENCES

3.1 Basic medicine (anatomy, cytology, physiology, genetics, pharmacy, pharmacology, toxicology, immunology and immunohaematology, clinical chemistry, clinical microbiology, pathology)

3.2 Clinical medicine (anaesthesiology, paediatrics, obstetrics and gynaecology, internal medicine, surgery, dentistry, neurology, psychiatry, radiology, therapeutics, otorhinolaryngology, ophthalmology)

3.3 Health sciences (public health services, social medicine, hygiene, nursing, epidemiology)

4. AGRICULTURAL SCIENCES

4.1 Agriculture, forestry, fisheries and allied sciences (agronomy, animal husbandry, fisheries, forestry, horticulture, other allied subjects)

4.2 Veterinary medicine

5. SOCIAL SCIENCES

5.1 Psychology

5.2 Economics

5.3 Educational sciences (education and training and other allied subjects)

5.4 Other social sciences [anthropology (social and cultural) and ethnology, demography, geography (human, economic and social), town and country planning, management, law, linguistics, political sciences, sociology, organisation and methods, miscellaneous social sciences and interdisciplinary , methodological and historical S1T activities relating to subjects in this group. Physical anthropology, physical geography and psychophysiology should normally be classified with the natural sciences].

6. HUMANITIES

6.1 History (history, prehistory and history, together with auxiliary historical disciplines such as archaeology, numismatics, palaeography, genealogy, etc.)

6.2 Languages and literature (ancient and modern)

6.3 Other humanities [philosophy (including the history of science and technology) arts, history of art, art criticism, painting, sculpture, musicology, dramatic art excluding artistic "research" of any kind, religion, theology, other fields and subjects pertaining to the humanities, methodological, historical and other S1T activities relating to the subjects in this group]