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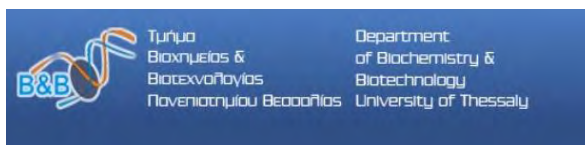
Generation and characterization of an immortalised human brain vascular pericyte cell line

Παραγωγή και χαρακτηρισμός αθάνατης κυτταρικής σειράς ανθρώπινων εγκεφαλικών αγγειακών περικυττάρων.

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Abstract

Malignant diffuse gliomas are the most common subtype of primary CNS neoplasms and their nature is rather aggressive. Showing clear glial differentiation characteristics, they are highly invasive tumors and neurologically destructive, considered to be among the deadliest of the human cancers. Glioblastoma multiforme (GBM) is the most lethal manifestation of all glioma types. Despite intense investigation of disease's biology most patients pass away within 15 months of diagnosis. They are highly heterogeneous entities, comprised of many different cellular types, with recently discovered self-renewing, tumorigenic GBM cancer stem-like cells being on top and conferring resistance to therapies and recurrence of the disease at the same time. These CSCs reside within organized microvascular domains called niches where they are protected from environmental insults. Thus, it seems promising that disruption of niche's function and formation might prove an effective therapeutic target. Among niche components, pericytes, seem to play a key role in tumor invasiveness and metastasis. The epithelial-to-mesenchymal transition (EMT) molecular program, apart from its prominent role in developmental processes and wound healing, seems to also facilitate tumor invasiveness and metastasis. GBM associated pericytes have been shown two express at least two EMT factors (Slug and Twist) whereas pericytes in normal brain areas do not show signs of expression of EMT related factors. The primary goal of this study was to generate an immortalized brain vascular cell line. Immortalization was achieved using a lentiviral construct carrying the SV40 large T antigen and the RFP gene. Immortalization of cells is a useful tool for in vitro studies, as it is known for a long time now that cells do not proliferate indefinitely making

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necessary the re-establishment of fresh cultures. A process that can prove quite tedious. Next goal as to define whether immortalization caused any phenotypic change by detecting the presence of some pericyte specific markers (CD146, NG2, PDGFRβ). Both primary and immortalized populations of cells equally expressed these markers confirming no sign of phenotypic alteration. Finally, we wished to examine the expression profiles of EMT related molecular markers (Slug, Snail, Twist, E-Cadherin, N-Cadherin, and Vimentin) in mRNA and protein level for both populations of cells, primary and immortalized. Primary and immortalised HBVPs were found to express small amounts of an EMT protein marker, Snail being clearly distinguished from GBM-associated pericytes which express Slug and Twist at least. N-Cadherin and Vimentin, mRNAs and proteins, were also detected in both cell populations. This cell line can be used in studies to assess the true nature of the functional network between GBM cells and -their secreted factors- and pericytes in the basis of putative EMT-driven metastasis and invasion.

Περίληψη

Τα κακοήθη διάχυτα γλοιώματα αποτελούν τον πιο κοινό υπότυπο πρωτογενών νεοπλασμάτων του ΚΝΣ και η φύση τους είναι ιδιαίτερα επιθετική. Έχοντας εμφανή χαρακτηριστικά διαφοροποιημένων κυττάρων γλοίας, είναι άκρως επιθετικοί όγκοι και οδηγούν σε καταστροφή του νευρικού συστήματος. Έτσι, συγκαταλέγονται στη κατηγορία με τους πιο θανατηφόρους καρκίνους του ανθρώπου. Το πολύμορφο γλοιβλάστωμα (glioblastoma multiforme, GBM) αποτελεί την πιο θανάσιμη μορφή όλων των τύπων γλοιώματος. Παρά την εκτενή μελέτη της βιολογίας του γλοιβλαστώματος, οι περισσότεροι ασθενείς αποβιώνουν μέσα σε 15 μήνες από τη στιγμή της διάγνωσης. Είναι όγκοι ιδιαίτερα ετερογενής και απαρτίζονται από πολλούς διαφορετικούς κυτταρικούς πληθυσμούς. Στη κορυφή της ιεραρχίας των κυττάρων που συγκροτούν το γλοιβλάστωμα, εντοπίζονται τα προσφάτως ανακαλυφθέντα καρκινικά κύτταρα που ομοιάζουν με βλαστικά κύτταρα και διαθέτουν ικανότητα αυτοανανέωσης. Σε αυτή την ομάδα κυττάρων αποδίδεται η αντοχή στις υπάρχουσες θεραπείες καθώς και ο υποτροπιασμός των ασθενών σε αρκετές περιπτώσεις. Αυτά τα καρκινικά βλαστικά κύτταρα εντοπίζονται μέσα σε περιοχές υψηλής αγγείωσης στο ευρύ μικροπεριβάλλον του όγκου, όπου προστατεύονται από περιβαλλοντικά ερεθίσματα. Συνεπώς η δομική και λειτουργική αποδιοργάνωση της περιοχής αυτής ίσως αποτελεί μία πολλά υποσχόμενη θεραπευτική προσέγγιση. Ανάμεσα στα λοιπά συστατικά στοιχεία του καρκινικού μικροπεριβάλλοντος, τα περικύτταρα (human brain vascular pericytes, HBVPs) παίζουν σημαντικό ρόλο στην καρκινική μετάσταση. Ο μοριακός μηχανισμός επιθηλιακής-μεσεγχυματικής μετάβασης (epithelial-to-mesenchymal transition, EMT), εκτός από τον

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προφανή του ρόλο σε αναπτυξιακές διαδικασίες και στην επιδιόρθωση τραυμάτων, φαίνεται να λειτουργεί και στα πλαίσια της διεισδυτικότητας των καρκινικών κυττάρων. Τα ¨καρκινικά περικύτταρα¨ ενός γλοιβλαστώματος εκφράζουν τουλάχιστον δύο ΕΜΤ παράγοντες (Slug και Twist) σε αντίθεση με τα φυσιολογικά περικύτταρα στα οποία δεν έχει παρατηρηθεί κάτι τέτοιο. Ο σκοπός αυτής της εργασίας ήταν η παραγωγή μίας αθάνατης σειράς εγκεφαλικών αγγειακών περικυττάρων. Αυτό επετεύχθη με μία ρετροϊκή κατασκευή που έφερε το γονίδιο του μεγάλου Τ αντιγόνου του ιού SV40 και ως γονίδιο αναφοράς το RFP. Οι αθάνατες κυτταρικές σειρές αποτελούν σημαντικό εργαλείο για in vitro μελέτες καθώς είναι γνωστό εδώ και αρκετό καιρό ότι τα κύτταρα παρουσιάζουν περιορισμένο πολλαπλασιαστικό δυναμικό, καθιστώντας αναγκαία την δημιουργία νέων καλλιεργειών από την αρχή. Μία διαδικασία που ενδέχεται να είναι αρκετά δύσκολη. Επόμενος στόχος της εργασίας ήταν να διαπιστωθεί εάν η διαδικασία της αθανατοποίησης των περικυττάρων οδήγησε σε τυχόν φαινοτυπικές αλλαγές μέσω της ανίχνευσης της έκφρασης συγκεκριμένων περικυτταρικών δεικτών (CD146, NG2, PDGFRβ). Τόσο τα πρωτογενή όσο και τα αθάνατα περικύτταρα εξέφραζαν εξίσου τους παραπάνω δείκτες χωρίς να διαφαίνεται κάποια φαινοτυπική αλλαγή. Τέλος, θέλαμε να εξετάσουμε το προφίλ έκφρασης, τόσο σε επίπεδο mRNA όσο και σε πρωτεϊνικό επίπεδο, πρωτογενών αλλά και αθάνατων περικυττάρων ως προς μία σειρά μοριακών δεικτών (Slug, Snail, Twist, E-καδερίνη, N-καδερίνη, and βιμεντίνη) που σχετίζονται με τη διαδιακασία ΕΜΤ. Και οι δύο κυτταρικοί πληθυσμοί φάνηκε να εκφράζουν μικρές ποσότητες ενός μόνου ΕΜΤ παράγοντα, του Snail. Γεγονός που διαφοροποιεί τα περικύτταρα αυτά από τα ¨καρκινικά περικύτταρα¨ που εκφράζουν

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τουλάχιστον δύο, τους Slug και Twist σε σημαντικές ποσότητες. mRNA των γονιδίων Ν-καδερίνης και βιμεντίνης όπως επίσης και οι αντίστοιχες πρωτεΐνες ανιχνεύθηκαν στους υπό μελέτη, δύο, κυτταρικούς πληθυσμούς. Η αθάνατη κυτταρική σειρά εγκεφαλικών αγγειακών περικυττάρων μπορεί να χρησιμοποιηθεί σε έρευνες για την αποσαφήνιση του δικτύου λειτουργικής αλληλεπίδρασης μεταξύ των καρκινικών κυττάρων του γλοιβλαστώματος -όπως επίσης και των διαλυτών παραγόντων που εκκρίνουν- και των καρκινικών περικυττάρων στα πλαίσια μίας πιθανής, εξαρτώμενης από παράγοντες ΕΜΤ, καρκινικής μετάστασης.

INTRODUCTION

1.1 Gliomas: Classification and Molecular Pathology

Primary brain tumors represent a large and quite heterogeneous group of neoplasms which originate from many different cell lineages although the true cellular origins demand further investigation. The classification system of these CNS neoplasms has been modulated on evidences coming from extensive histopathological studies. The last few decades though, new molecular criteria, which underline the biological basis of these malignancies more aptly, have been established in order to structure an accurate classification system. And those striking advances have been made in regard to malignant glioma and medulloblastoma, the most common tumors of adults and children, respectively (Huse et al. 2010).

Gliomas are the most common primary tumors of the central nervous system (CNS) and account for the 2% of the total human malignancies. The common gliomas that affect the cerebral hemispheres of adults are termed "diffuse gliomas" because of their innate tendency to infiltrate throughout the brain (Lim et al. 2011).

These tumors show evidences of glial differentiation (astrocytes and oligodendrocytes) in a histological basis. They presumably derive from either mature glia or their less differentiated precursors. Gliomas are divided into distinct classes according to histological features of malignancy such as: proliferation rate, cellular pleomorphism, vascular activity and necrosis (Maher et al. 2001).

The World Health Organization (WHO), according to its most recent upgrade which took place in 2007, classifies gliomas into four different grades according to the degree of malignancy (Louis et al., 2007):

1. Grade I: Grade I glial tumors are benign, slow-growing, and well-circumscribed tumors that are typically curable with resection.
2. Grade II: Grade II glial tumors infiltrate into normal brain tissue and exhibit moderate proliferative feature. These tumors have the potential to undergo malignant transformation (~70%) within 5 to 10 years from diagnosis.
3. Grade III and IV: Grades III and IV tumors are classified as malignant gliomas, with grade IV being extremely aggressive with hallmarks of vascular and uncontrolled cellular proliferation, diffuse infiltration, and necrosis (Jones and Holland 2011).

Diffuse gliomas are classified, according to the cell lineage they may be arising from, into: astrocytomas, oligodendrogliomas, and tumors with morphological features of both astrocytes and oligodendrocytes, termed oligoastrocytomas. Astrocytic tumors are subsequently graded as pilocytic astrocytoma, grade I; astrocytoma, grade II; anaplastic astrocytoma, grade III; and glioblastoma, grade IV. Grade I and II low grade astrocytomas are slow growing and less aggressive tumors while grade III and IV high grade gliomas are malignant tumors characterized by high mitotic activity (grade III) and induction of necrotic tissue and/or angiogenic activity (grade IV). Oligodendrogliomas and oligoastrocytomas are graded as grade II or anaplastic, grade III (Maher et al. 2001, Huse et al. 2010).
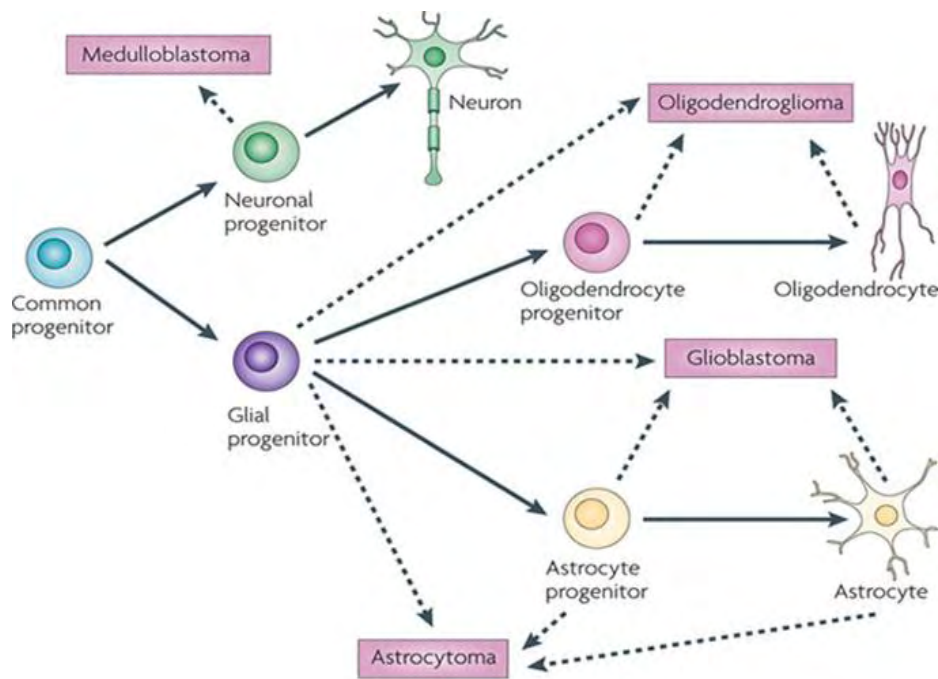
Fig.1: Precise initiating cell lineage for the glioma variants and medulloblastoma remain largely unknown, likely candidates (dashed arrows) are indicated (Huse and Holland 2010).

Glioblastoma Multiforme (GBM, grade IV) is the most lethal manifestation of the gliomas accounting for 69% of all incident cases of astrocytic and oligodendroglial tumors with a median survival of 15 months, even at best therapeutic approach. Two clinical subtypes for GBM have been identified. Thus, glioblastomas can either be "primary'' or "secondary''. Primary or "de novo" GBMs arise without any prior clinical or histological evidence of a less malignant precursor lesion. They commonly affect older patients (mean age of 62 years old). Secondary GBMs slowly develop from a lower grade glioma. The precursor lesion might either be a low grade astrocytoma (WHO grade II) or an anaplastic astrocytoma (WHO grade III) and typically affect younger patients (mean age of 45 years old) (Lim et al. 2011, Ohgaki and Kleihues 2007).

Primary and secondary GBMs are morphologically and clinically indistinguishable in the basis of the poor prognosis in their respective target group of patients. However, recent molecular advances have shown quite distinct genetic profiles for the two GBM forms as well as new potential molecular subclasses within each of the two major categories (Furnari et al. 2007).

Compared to primary glioblastomas, the secondary glioblastomas constitute only the 5% of the cases at a population level. These might be explained by the fact that patients succumb to the disease before any progression to secondary forms occurs (Ohgaki and Kleihues 2007).
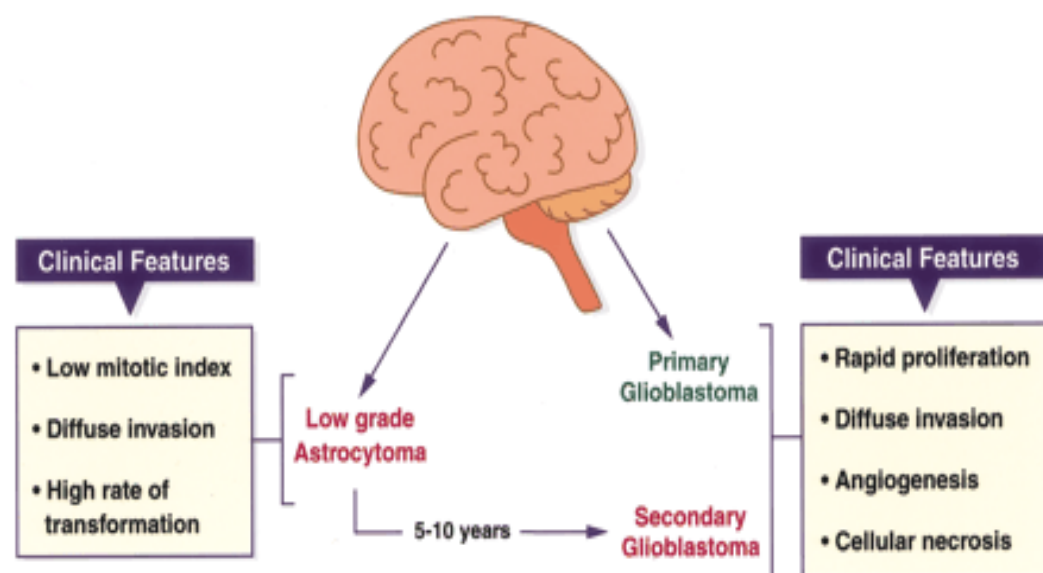

Fig.2: Two pathways to GBM. GBM can develop over 5–10 yr from a low-grade astrocytoma (secondary GBM), or it can be the initial pathology at diagnosis (primary GBM). The clinical features of GBM are the same regardless of clinical route (Maher et al. 2001)

Although most of the glioma cases are sporadic, genetics seem to play a key role in the pathogenesis of the disease. 5% of glioma patients present a familial history associated with some rare genetic syndromes, such as, neurofibromatosis types 1 and 2, the Li-Fraumeni syndrome, Turcot's syndrome (Wen and Kesari, 2008).

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A vast collection of well characterized genetic alterations in pathways governing cellular survival, cellular proliferation, invasion and angiogenesis contribute to the malignant phenotype of gliomas. It has been reported that the most frequently mutated genes in human GBM include, P53, PTEN, EGFR, CDKN2A/2B and NF1, indicating a possible role for NF1 as a glioblastoma tumor suppressor gene (Lim et al. 2011).

- ***Secondary GBM***

Initiation pathways:

TP53.Genetic alterations of the p53 gene are observed in both low and high grade astrocytomas, implicating a possible role in early tumor formation. P53 is a tumor suppressor that acts as a transcription factor regulating G1 to S cell cycle progression and apoptosis. Alterations include mutations in the DNA binding region of the protein. Its role in gliomagenesis is also corroborated by the observation that patients with Li-Fraumeni syndrome –a familial cancer syndrome characterized by germline mutation of the TP53 gene- are predisposed to development of CNS tumors, including astrocytomas (Zhu and Parada 2002).

Receptor tyrosine kinases and RAS. RTK signaling pathways, activated by diffusible growth factors, confer control of cell survival/proliferation/motility to normal cells. The small GTP binding protein RAS, is an important downstream effector that activates at least three downstream cascades: RAF-MEK-ERK (proliferation), PI3K-AKT (survival), CDC42-RAC-RHO (motility). Activating, constitutive mutations of RTKs are commonly found in cancer cells because they render them independent of exogenous activation by growth factors. Elevated

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expression of platelet derived growth factor (PDGF) and its receptor (PDGRF) are found in every grade of astrocytoma indicating that this axis might be involved in astrocytoma development. On the other hand, no RAS mutations have been identified in human astrocytomas. Instead, RAS can be activated indirectly by upregulation of the PDGF/PDGFR axis, loss of function of the NF1 proteins which is a negative regulator of RAS activity (RAS mediated MAPK and PI3K cascades are found in both neurofibromatosis type 1 mutant-associated and sporadic astrocytomas) or through other RAS activating mechanisms (Zhu and Parada 2002).

Progression pathways:

pRB. RB also acts as a guardian of the cell cycle by controlling the G1-S transition. In quiescent cells, RB proteins are hypophosphorylated and they sequester the E2F transcription factors. Upon mitotic activation, RB is partially phosphorylated by CDK4/6 and releases the E2F factors which activate genes required for the G1-S transition. Cyclin dependent kinase inhibitors (CKIs) provide positive regulation of this pathway. The INK4A CKI (known as p16^{INK4a}), encoded by the CDKN2A locus, inhibits the activation of cyclin D and CDK4/6 complex. This RB mediated regulatory circuit is important for astrocytoma progression and initiation as mutations in the INK4A/CDK4/RB axis (loss of RB or amplification of CDK4) are solely found in more than 80% of GBMs and in 50% of anaplastic astrocytomas (Maher et al. 2001)

- ***Primary GBM***

ARF(p14^{ARF})/INK4A(p16^{INK4a}). In primary GBM, disruption of the p53 pathway occurs mainly through the loss of the gene that encodes the ARF (p14ARF) or through amplification of MDM2 instead of direct deactivating mutation of the TP53 gene (secondary GBM). P53 loss of function results from amplification of the MDM2 gene, which as an ubiquitin ligase targets p53 for proteasomal degradation, or through loss of function of p14^{ARF} which antagonizes Mdm2 and is also encoded by the CDKN2A locus. The p16 pathway is also dismantled in primary GBM, mainly through homozygous deletions of the INK4A gene (40% of cases), phenomenon observed only in 4% of secondary GBM. Homozygous deletion of the CDKN2A locus leads to concomitant ablation of INK4A and ARF functions, therefore loss of RB and p53 pathways. This might explain the rapid manifestation of primary GBMs (Zhu and Parada 2002).

EGF/EGFR. Amplification of the gene encoding the epidermal growth factor receptor (EGFR) is found in 40% of primary GBMs. Additional to the amplification of the EGFR gene, intragenic rearrangements lead to a constitutively active receptor. EGFR amplification is related to INK4A gene mutations supporting the hypothesis that cooperation of the two altered pathways might contribute to tumor formation (Furnari et al. 2007).

PTEN. Loss of the long arm of the chromosome 10 is the most common genetic alteration associated with GBMs. One of the loci of this lost region is the PTEN, a protein and lipid phosphatase which acts as a tumor suppressor. Loss of PTEN is observed in more than 30% of primary GBM and rarely in secondary GBM. One of the PTEN substrates is the PI3K

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kinase and thus PTEN acts as a negative regulator of the PI3K/AKT pathway which is involved in cell survival (Zhu and Parada 2002).
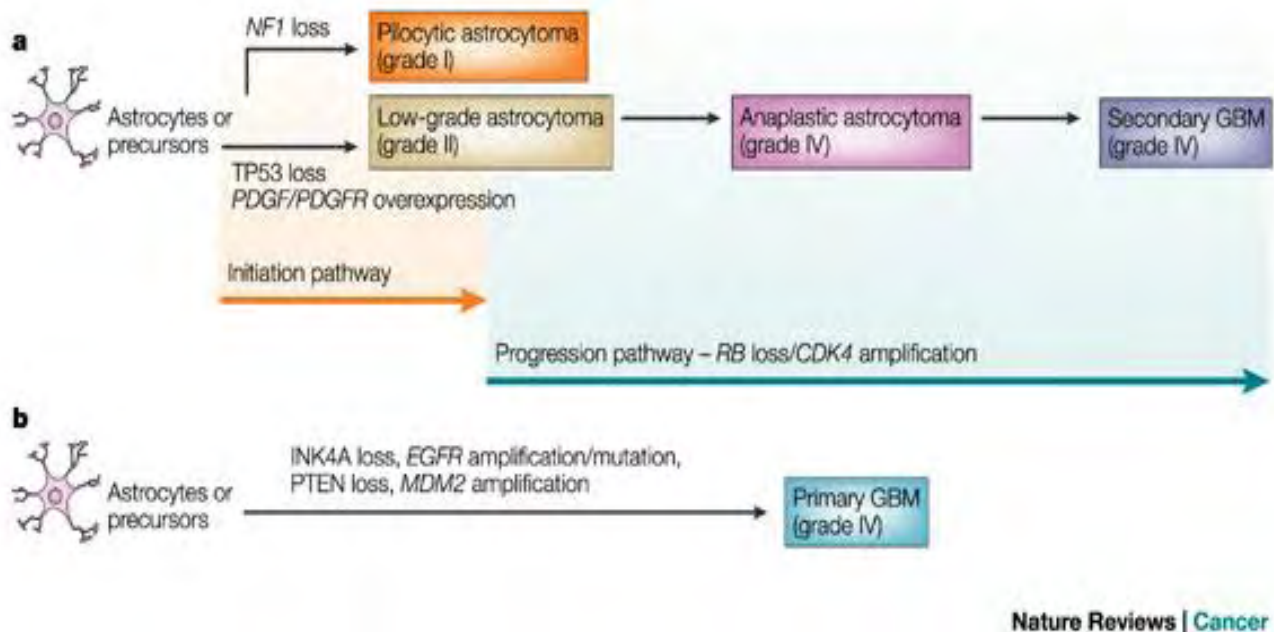

Fig.3: Genetic pathways involved in the development of primary and secondary glioblastoma (Zhu and Parada 2002).

1.2 Clues to the glioma initiating cell

Traditional views hold that gliomagenesis is attributed to the malignant transformation of the differentiated glial components of the CNS, that is, astrocytes and oligodendrocytes. This theory was based on the belief that brain is incapable of post-natal neurogenesis and that glial cells are the only ones capable of proliferation (Chesler et al. 2013).

Numerous studies have recently proven the existence of multipotent neural stem cells (NSCs) and lineage restricted progenitor cells in the CNS of postnatal mammalian brain revealing a previously unknown aspect of plasticity. NSCs are self-renewing, multipotent cells with astrocytic features that can differentiate into tissue components of the brain. These

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cells have been located in the human dentate gyrus, subcortical white matter and the subventricular white matter (Nduom et al. 2012).

Many studies have provided evidence for the existence of a stem-like subpopulation in a tumor which is considered responsible for its tumorigenicity and these cells are called Cancer Stem Cells (CSCs) and share many common properties with NSCs. Experiments were able to determine that there were in fact cells within gliomas which exhibited characteristics of normal NSCs such as the ability to self-renew, proliferate and produce a variety of progeny. These cells were also shown to carry genetic aberrations and could generate orthotopic tumor xenografts when implanted in the brains of mice. Based on the evidence above, the Cancer Stem Cell theory for gliomagenesis started to gain ground. Based on the similarities beteween NSCs and glioma initiating cells (GICs) it has been postulated that GICs are NSCs derivatives Moreover, much of the therapy resistance and the ability to regenerate when the bulk of the tumor has been treated seems to rest within this glioma stem-like subpopulation (Nduom et al. 2012).

Studies have focused on the identification of molecular markers which facilitate the isolation of NSCs and CSCs within the heterogeneous environment of a tumor. Thus, the CD133 marker (prominin I) has been efficiently used. While it was originally postulated that only CD133+ cells were able to be tumorigenic, recently it was shown that CD133- contain considerable tumorigenic capacity (Fatoo et al. 2011, Nduom et al. 2012).

Research focuses on the nature of the tumor initiating cell (TIC) which is the first genetically aberrant that can initiate the process of carcinogenesis. Overtime, TIC, acquires multiple genetic insults and

becomes a cancer stem-like cell. Studies have shown that neural progenitor cells, neural stem cells as well as mature astrocytes, under the effect of genetic alterations, such as EGFR amplification and deletion of the INKK4a/ARF locus, lead to the development of GBM-resembling tumors and thus could be the cell(s) of origin (Nduom et al. 2012).
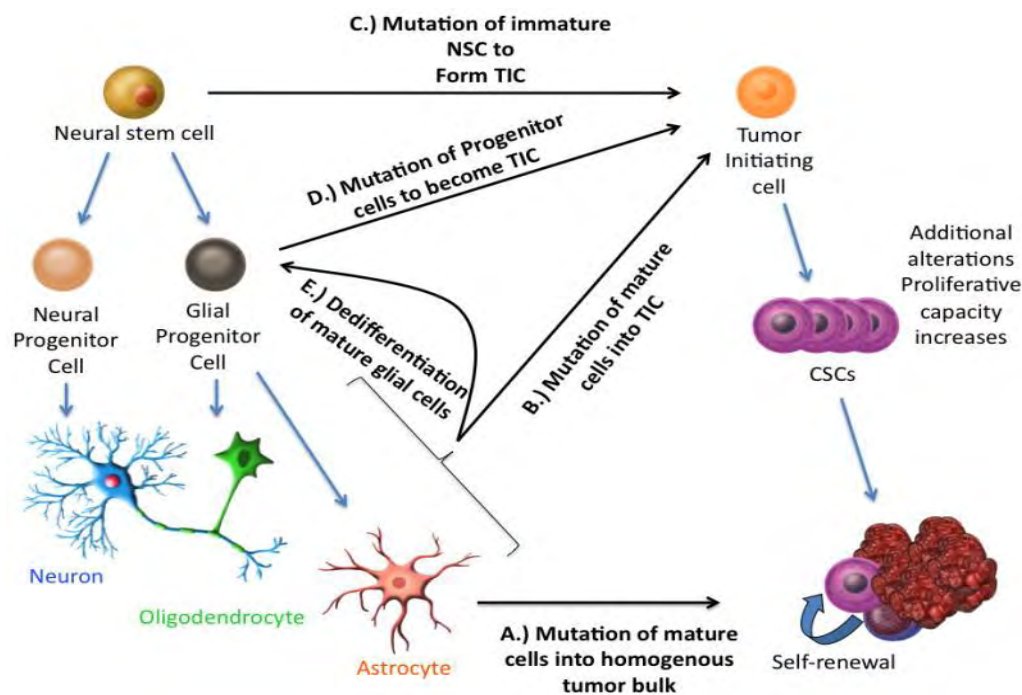

Fig. 4: An overview of the various pathways leading to the genesis of the tumor initiating cell and the subsequent tumor development (Nduom et al. 2012)

1.3 Glioblastoma microenvironment

It has long been recognized that normal stem cells, including NSCs, exist within dynamic and heterogeneous specialized micro domains, called perivascular niches (PVN) in close proximity to the endothelial cells. Direct cell contacts (e.g. Notch signaling) and secreted signals (e.g. VEGF), originating from the niche various cellular components, maintain stem cell homeostasis by regulating the balance between self-renewal and differentiation (Gilbertson et al. 2007).

Angiogenesis is a process tightly related to tumor progression and the formation of a rather irregular vasculature, which serves the inordinate demands for oxygen and nutrients, is a hallmark of GBM. Apart from satisfying the hyperbolic ''feeding demands'' of GBM, this excessively disorganized vascular bed, contributes to the formation of an abnormal PVN which maintains the GBM stem-like cells and resembles the niche of the normal neural stem cells in the SVZ of the brain. However, niche dependence between normal NSCs and GBM CSCs differs greatly (Gilbertson et al. 2007).

Indeed the brain tumor microvasculature forms rather aberrant niches where the Nestin^{+}/CD133^{+} cancer cells, which include the CSC fraction, are maintained. This cells, tend to be located in close proximity to tumor capillaries and the self-renewal and proliferation capacity of the Nestin+/CD133+ is maintained in vitro by factors secreted by endothelial cells (Calabrese et al. 2007). On the other hand, CSCs have been shown to secrete angiogenic factors, such as VEGF, that promote recruitment and formation of tumor blood vessels under the necrotic and hypoxic conditions of GBM (Bao et al. 2006). Therefore, an important, bidirectional functional interplay between GBM stem-like cells and the endothelial cells of the aberrant PVN has been established. The rupture of these perivascular niches, using antiangiogenic factors, ablates the fraction of CSCs and arrests tumor growth (Calabrese et al. 2007).

However clinical experience with antiangiogenic factors has taught that there are multiple, diverse, compensating mechanisms by which tumors develop resistance, such as transdifferentiation of tumor cells into endothelial cells. Targeting the structure of niche by trying to abrogate its

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formation may not be practical. Instead, targeting the complex niche function by disrupting the communication between its components may have more efficient therapeutic effects (Brooks et al. 2013).

The niche presents multiple functions: maintains stem cell phenotype by blocking differentiation, promotes tumor cell proliferation, protects against environmental insults such as radiation and chemotherapy and facilitates the infiltrative spread of GBM by providing a platform for the dissemination of the glioblastoma cells. The plethora of PVN functions, is attributed to the orchestrated activities of a rather diverse network of cells. Similar to the neurogenic niche of normal NSCs, the GBM niche is comprised of endothelial cells, pericytes, astrocytes as well as immune cells such as microphages/microglia, ependymal cells, extracellular-matrix components and cell adhesion signaling. (Brooks et al. 2013).
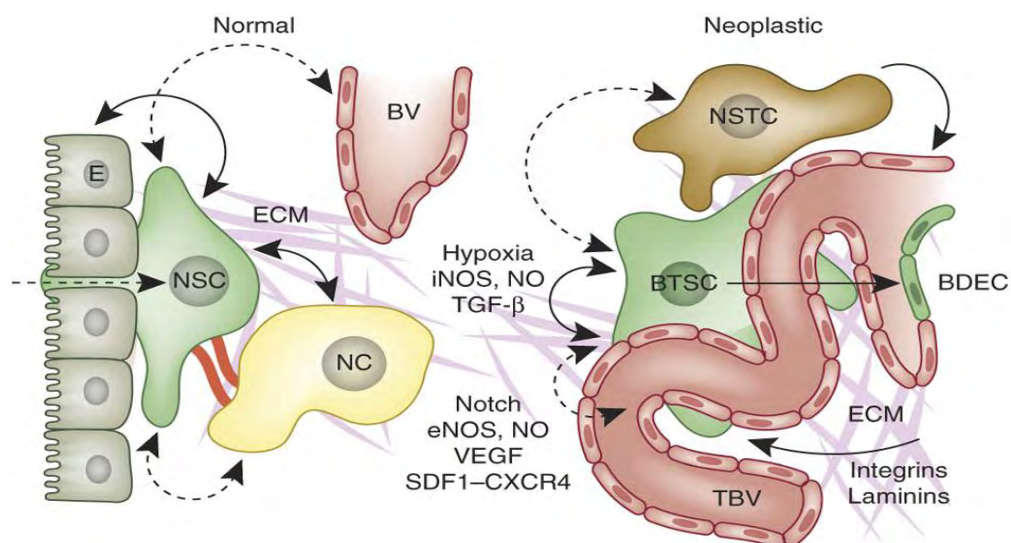

Fig.5: Communication between cells in the perivascular niche. Neural stem cells (NSCs) rely on a series of signals between the extracellular matrix (ECM), blood vessels (BV), ependymal cells (E) and other niche cells (NC) to promote their maintenance. BTSCs rely on similar interactions in their abnormal perivascular niche, which also consists of ECM, non-stem tumor cells (NSTC) and atypical tumor blood vessels (TBV). BTSC plasticity toward an endothelial lineage and incorporation of these BTSC-derived endothelial cells (BDECs) into the vasculature may also contribute to the perivascular niche (Hjelmeland et al. 2011).

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1.4 Epithelial to Mesenchymal Transition (EMT) and Tumor Progression

EMT is the process by which, cells lose their epithelial characteristics, including their polarity and their specialized cell-cell contacts, and acquire a migratory behavior, allowing them to move away from their epithelial cell community and integrate into the surrounding tissue, even at remote locations. In other terms, cell adopt a more mesenchymal phenotype (Zavadil et al. 2005).

Epithelial cells typically form sheets, tubes or vesicles in which the cells acquire an apical-basal polarity. In these structures, cells intimately associate through specialized cell-cell contact structures: tight junctions, adherens junctions, and desmosomes. One of the EMT early events is the disorganization of the tight junctions which results in the redistribution of the proteins from which they are composed (occludins, claudins, zonula occludens) along with disruption of the polarity complex and initiation of cytoskeleton reorganization. During EMT, adherens junctions, comprised of E-cadherin adhesion receptor, disassemble and the actin cytoskeleton is reorganized into actin stress fibers anchored to focal adhesion complexes. Finally, EMT leads to the downregulation of the desmosome components and the disassociation of these structures. The acquisition of a mesenchymal phenotype is characterized by the expression of mesenchymal cytoskeletal proteins, such as vimentin, and the deposition of ECM components, including collagens and fibronectin. These components stimulate integrin signaling and induce formation of focal adhesion complexes which facilitate cell motility. Concomitant upregulation of N-cadherin, renders cells motile and invasive. At the same time, increased activity of ECM proteases allows cells to degrade ECM

proteins, escape from epithelial structures and migrate easier (Xu et al.2009).

EMT is normally involved in many processes during embryogenesis as well as in wound healing procedures. This multilateral process results from changes in the cell's transcriptional programme, where epithelial gene expression is represed whereas expression of mesenchymal genes is activated. Several transcriptional suppressor families regulate EMT, including the zinc-finger proteins Snail1 and Snail2, the two-handed zinc-finger δEF1 family factors Zeb1 and SIP1/Zeb2, and the basic HLH factors TWIST and E12/E47 (Creighton et al. 2013).

Many cellular process are co-opted by cancer cells to their own advantage and there is much evidence that, EMT, being one of these processes can in fact aid tumor invasion and metastasis. EMT would enable tumor epithelial cells to lose cell polarity and cell-cell adhesive interactions and junctions, allowing them to escape the primary tumor. Recently, EMT has been associated with the CSCs which are believed to be highly tumorigenic, since these cells have been found to express EMT markers (Creighton et al. 2013).

Despite the importance of glioma invasion, little is known about how this complex phenotype is regulated so that effective anti-invasion therapies can be developed. By contrast, the process by which human epithelial cancers, or carcinomas, acquire an invasive phenotype has been more extensively characterized at both the cellular and molecular levels where the contribution of the EMT process has been clearly indicated. However, recent recognition of mesenchymal change in glioblastoma and its association with more aggressive clinical phenotypes and poor outcome

suggests that mechanisms that promote EMT in carcinoma may be of great clinical relevance in GBM (Phillips et al. 2006, Mikheeva et al. 2010). In fact, Mikheeva et al., identified TWIST1 as a putative regulator of mesenchymal change and invasion in GBM (Mikheeva et al. 2010).

1.5 The role of pericytes in glioma vasculature

Blood vessels are composed of two interacting cell types: Endothelial cells which form the inner lining of the vessel wall, and perivascular cells – referred to as pericytes, vascular smooth muscle cells or mural cells – which envelop the surface of the vascular tube (Bergers and Song 2005).

Vascular pericytes, normally of mesenchymal origin, play critical roles in various physiological contexts, including support of vascular structure and function, maintenance of blood-brain barrier, facilitation of vessel maturation and initiation of vessel sprouting. The significance of pericytes in blood vessel formation is reflected by the fact that when vessels lose their pericytic coverage, they become hemorrhagic and hyperdilated. Pericytes and ECs communicate through direct physical contact (adhesion plaques) and reciprocal signaling to maintain vessel integrity and function (Cheng et al. 2013, Bergers and Song 2005).

Pericytes together with other cell populations and ECM components build up the microenvironment where tumor cells reside and proliferate, the tumor stroma. Despite their importance as tumor stroma ingredients little is known about their origin, recruitment and interaction with other stromal or tumor cells. Angiogenesis in tumors leads to a chaotic vasculature with irregularly shaped and leaky vessels that are often unable to support efficient blood flow. Just as tumor endothelial cells

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differ from the normal, quiescent endothelium, tumor pericytes also differ from normal pericytes. In general, pericytes in tumors appear to be more loosely attached to the vasculature, their cytoplasmic processes can extend into the tumor parenchyma, they also seem to be less abundant in some tumor tissues in comparison to the respective normal tissue and finally can change their pericyte expression profile. Pericyte coverage of blood vessels is also dependent on the tumor type: glioblastomas and mammary carcinomas exhibit a more dramatic reduction in pericyte density when compared to the respective normal tissues. Although, pericyte density reduction produces a dysfunctional vascular bed, only reductions >90% are lethal. This observation suggests that even small numbers of pericytes in tumors can still be functional and important for vessel stability and endothelial cell survival (Bergers and Song 2005, Armulik et al. 2011).

The ontogeny of pericytes is a matter of great debate. The majority of the mural cells in all CNS parts are neural crest derived and new pericytes develop mainly by proliferation of pre-existing ones, given the lack of immature mesenchyme in developing CNS. However, in coelomic organs, mesothelial cells undergo EMT, delaminate and migrate into organs to produce mesenchymal components such as fibroblasts, vSMCs, and most likely pericytes. In spite of this common mesothelial origin of the pericytes in organs such as heart, liver and gut, different signaling pathways govern their recruitment to the respective periendothelial location. (Armulik et al. 2011).

The origin and subsequently the contribution of pericytes in pathologic conditions, such as cancer, is a matter that needs further elucidation. Focusing on GBM, research has been trying to figure the true origin of pericytes on tumor blood vessels. Tumor pericytes are thought to be derived from their progenitors from their surrounding normal tissue or from the bone marrow derived cells residing in tumors after treatments. It is noteworthy the fact that GSCs have the ability to undergo mesenchymal differentiation and share characteristics with NSCs that present the potential of transdifferentiating into pericytes. Therefore, it can be concluded that GSCs may possess the capacity of differentiating into pericytes which support tumor growth. Indeed, Cheng et al provided evidence that GSCs remodulate their microenvironment by contributing pericyte compartments of the neovasculature. Moreover, elimination of this G-pericytes, disrupted vessels and inhibited tumor growth (Cheng et al. 2013).

1.6 Immortalization of cells

Normal human somatic cells have a limited capacity to replicate in culture, even under conditions that appear to satisfy their nutritional and mitogen requirements. These cells proliferate initially but eventually enter a state of permanent growth arrest termed senescence. This state, has been alternatively described as Hayflick Limit.

Telomere shortening provided the first molecular explanation for why many cells cease to divide in culture. Dysfunctional telomeres trigger senescence through the p53 pathway. This response is often termed telomere-initiated cellular senescence. Some cells undergo replicative senescence independently of telomere shortening. This senescence is due

to stress, the nature of which is poorly understood. It increases p16 expression and engages the p16–retinoblastoma protein (pRB) pathway. This response is termed stress-induced or premature senescence, stasis or M0 (mortality phase 0). The senescence cell phenotype, caused by dysfunctional telomeres and/or strong mitogenic signals and/or non telomeric damage and/or genotoxic stress, is characterised by metabolically active, resistance to apoptosis signals and often acquire altered gene expression patterns (Campisi and d'Adda di Fagagna 2007).

As primary cells reach senescence after a limited number of population doublings, researchers need to re-establish fresh cultures. A process that can be rather tedious. In order to have consistent material throughout a research project, researchers need primary cells with an extended replicative capacity, in other words, immortalized cells. The ideal immortalized cells are cells the ones capable of extended proliferation, but also possess similar or identical genotype and phenotype to the primary ones. In fact, immortalized cells, malignant or not, have been found to maintain stable telomere length (Stewart and Weinberg 2002).

Several methods have been introduced for immortalizing mammalian cells in culture conditions. One method is to use viral genes, such as the simian virus 40 (SV40) T antigen, to induce immortalization. SV40 T antigen has been shown to be the simplest and most reliable agent for the immortalization of many different cell types and the mechanism of SV40 T antigen in cell immortalization is relatively well understood. The large T antigen exerts its immortalizing effect by abrogating p53 and pRB tumor suppressor pathways while can also induce telomerase activity in some cells (Zhu et al. 1991, Foddis et al., 2002).

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The most recently discovered approach to cell immortalization is through the expression of Telomerase Reverse Transcriptase protein (TERT). This protein is normally inactive in most somatic cells, but when hTERT is exogenously expressed, the cells are able to maintain sufficient telomere lengths to avoid replicative senescence. Analysis of several telomerase immortalized cell lines has verified that the cells retain their parental phenotype and genotype (Wolbank et al. 2011). In mammary epithelial cells and keratinocytes, maybe TERT overexpression is not sufficient to immortalize cells and it must be combined with a supplementary process, such us silencing of the p16 signaling axis (Kiyono et al., 1998).

Other, less common immortalizing techniques, include the c-myc oncogene and the HPV (Human Papilloma Virus) E6/E7 proteins. Myc oncogene induces telomerase in both normal human mammary epithelial cells (HMECs) and normal human diploid fibroblasts. Myc increases expression of the putative human telomerase catalytic subunit gene hEST2 (hTRT/TP2), the limiting subunit of telomerase, and both Myc and hEST2 can extend the life span of human mammary epithelial cells (HMECs) (Wang et al. 1998). E6 and E7 Papilloma virus protein co-operate and manage to induce immortalization by dismantling the p16 and pRB pathways and inducing telomerase activity (Klingelhutz and Roman 2012).

Aim of the project

Preliminary study has shown that EMT factors Slug and Twist are expressed in glioma associated pericytes but not in pericytes of healthy brain vasculature (unpublished results). Under this perspective, and based on the complex multicellular composition of the GBM niche where pericytes are found, it is important to address what are the effector cells or signaling molecules which induce expression of the EMT factors in GBM associated pericytes and moreover, what are the effects of EMT upregulation in pericyte functional characteristics. As it was mentioned above, the restricted proliferative capacity of cells, hinders their in vitro use and the re-establishment of new cell cultures from parental tissue can become a rather tiresome procedure. The aim of this project was to generate an immortalised human brain vascular pericyte (HBVP) cell line using a lentiviral vector containing the SV40 large T-antigen whose immortalizing effects have been well studied. After that, the goal was, to address in mRNA as well as in protein level, a group of genes in order to compare the expression profiles of the primary and the immortalized HBVPs and validate whether indeed SV40 mediated immortalization retains the parental phenotype of the HBVPs as seen in other cases. The genes that were addressed, included some specific pericyte markers (CD146, NG2, PDGFRβ and α-SMA) - even though their expression is not pericyte exclusive (Bergers and Song 2005) - , a series of EMT factors (Slug, Snail, Twist) as well as E-Cadherin, N-Cadherin and Vimentin.

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2. MATERIALS AND METHODS

2.1 Immortalization protocol

The immortalization of the human primary brain vascular pericytes was performed using an **SV40 Large T-antigen - RFP - PURO** lentiviral vector (1×10^7 IFU/mL) purchased from Amsbio, according to manufacturer's instructions. Puromycin served as the resistance marker for selection.

Lentivectors are HIV-1 (Human Immunodeficiency Virus 1) derived plasmids, used to generate lentiviral particles (lentivirus) that can be transduced into almost all kinds of mammalian cell types or organs, including stem cells, primary cells and non-dividing cells both in vivo and in cell culture system. Particles stably integrate into transduced cells' genome for long term expression.

After immortalization, cells SV40 HBVPs were sorted with fluorescence-activated cell sorting (FACS) analysis into two populations: One with a high number of integrated viral genomes into the cell genome (HIGH population), showing high level of fluorescence, and one with a low number of integrated viral genomes (LOW population), presenting low level of fluorescence.
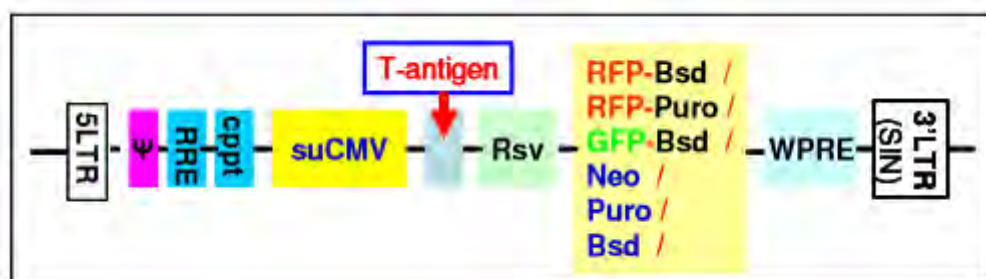
Lenti-SV40-RFP-PURO construct (Pre-made Lentiviral Particles for SV40 large T-antigen, Amsbio).

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2.2 Cell samples

Human Brain Vascular Pericytes were obtained from ScienCell™ (cat.number #1200).

Three different HBVP samples were used in the experiment:

1.   Primary HBVPs-Passage 6.

2.   HIGH Lenti-SV40-GFP-PURO HBVPs-Passage 15.

3.   LOW Lenti-SV40-GFP-PURO HBVPs-Passage 16.

For the purpose of the experiment, two T-75 flasks were used for each of the three different HBVP samples. Cells were cultured until full density in both flasks and afterwards one flask was used for mRNA isolation/cDNA synthesis/PCR and the other for Lysate Preparation/SDS-PAGE Western Blotting.

2.3 Cell culture and chemicals

The HBVPs of the current experiment were cultivated in T-75 flasks form CELLSTAR®. Flasks were coated before usage with poly-L-lysine solution (1:333). The basic growth medium was Pericyte Medium (PM, Cat.No. 1201) and it was supplemented with: FBS (Cat.No. 0010), PGS 100X (Cat.No. 1252) and Penicillin/Streptomycin Solution 100x (Cat.No. 0503). HBVPs were cultured normally in 10 mL of medium. All chemicals were purchased from ScienCell™. Cells were subcultured when they reached full confluency. After medium removal, HBVPs were washed with 5 mL of PBS (SIGMA-ALDRICH) and detached with 1 mL of 1X Trypsin EDTA (GE Healthcare). Cells were thoroughly resuspended in 2 mL of fresh medium and transferred into a new sterile Falcon tube. 250 µL of the cell solution were transferred into a new, poly-L-lysine coated T-75 flask containing 10

ml of fresh medium. The rest of the cell suspension was centrifuged at 2500 rpm for 5 min at 24oC. After medium removal, the cell precipitate was quickly resuspended in 1 mL of freezing buffer containing FBS (Cat.No. 0010) and 10% DMSO. 1 mL of freezing buffer plus HBVPs was aliquoted into two Eppendorf cups and cells were frozen at -80oC.

2.4 Cell pellet preparation

In order to prepare cell pellets, HBVPs were trypsinized with 1 mL of 1X Trypsin EDTA when they reached full density. After cells were detached, 2.5 mL of fresh full PM (supplemented with FBS, PGS, P/S) were added into the flask to bring cells into solution. The cell suspension was aliquoted into two, new, sterile Eppendorf cups and samples were microcentrifuged at 2.000 x g for 3 min. The supernatant containing PM was removed and pellets were stored at -80oC until use. The purpose of preparing two samples of cell pellets is, that one of them will be used for total mRNA isolation and the second one for protein lysate preparation.

2.5 Total mRNA isolation

Total mRNA isolation out of the cell pellets, was performed using the High Pure RNA Isolation Kit (ROCHE), according to manufacturer's instructions. After extraction, the total mRNA of each sample was quantified using the Nano Drop Spectrophotometer ND1000 and the respective Nano Drop Software 1000 3.8.1 (PEQLAB Biotechnologie GmbH).

2.6 cDNA synthesis

Complementary DNA was synthesized using the M-MLV Reverse Transcriptase (M170B 24228217, Promega) and the M-MLV 5X RT-Buffer (M531A 19847209, Promega).

Reverse Transcriptase is an enzyme which generates complementary DNA (cDNA) using an mRNA molecule as a template. A process called, Reverse Transcription. During the reverse transcription, an mRNA/cDNA helix is produced, where mRNA is later on cleaved by an RNaseH leaving a cDNA molecule ready for PCR application. Like polymerase, the RT enzyme, needs a primer to initiate the reverse transcription. There are three alternative primer methods:

1. Gene specific primers: Are used when the mRNA sequence is known.

2. Oligo d(T) primers: These primers contain a series of thymidine residues which bind to the poly(A) tail at the 3' end of an mRNA molecule. In these case, it may be challenging to get transcripts of the entire 5' end, depending on the length of the template.

3. Random primers: As indicated by their name, these primers consist of six to nine nucleotides put together in a random manner. Thus, the primers may anneal at several sites in an mRNA molecule and the reaction initiates from each site.

Oligo d(T) primers as well as Random primers can be used in case of an unknown mRNA sequence.

After the determination of the mRNA concentration, 5 μg from each sample were used for cDNA synthesis using random primers. All amounts are calculated for a final volume of 20 μL. The protocol was as follows:

1. RNA/primer mixtures:

- 13 µL (5 µg) of mRNA solution with RNase free water.
- 1 µL of dNTPs mixture.
- 1 µL of Random primer.

2. Incubation at 70oC for 10 min, and then on ice for 2 min. After cooling, samples are centrifuged briefly.

3. In each tube are added: 4 µL of 5X RT buffer and 1 µL of RT enzyme at last.

4. Samples are incubated at 42oC for 1 hour, with no mechanical agitation.

5. After incubation, samples are heated at 90oC for 10 min.

6. If more than 2 µg of mRNA are used, we have to act as follows:

- For 3 µg of mRNA we fill up to Vf=30 µL with RNase free water.
- For 4 µg of mRNA we fill up to Vf=40 µL with RNase free water.
- For 5 µg of mRNA we fill up to Vf=50 µL with RNase free water.

The cDNA can be stored at -20oC until further use.

2.7 Polymerase chain reaction

Polymerase Chain Reaction is one of the most pioneer applications in the field of Molecular Biology developed by Kary Mullis in the 1980s. PCR is based on the ability of DNA polymerase to synthesize a new strand of DNA, complementary to an offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide.

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This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies which are called amplicons.

A standard PCR requires some specific ingredients:

1. DNA template: The DNA template contains the target sequence which we wish to amplify and it must be lying between known regions of the dsDNA (flanks). Too much of the template results in non-specific amplification.

2. DNA polymerase: It is the enzyme that synthesizes new strands of DNA complementary to the target sequence. The most common polymerase, used in the majority of PCRs, is the Taq Polymerase which was originally isolated from Thermis aquaticus. All polymerases used in PCRs must be heat resistant.

3. Primers: A pair of oligonucleotides, each one complementary to one strand of the target sequence, which offer the necessary 3' –OH group for the Taq Polymerase in order to begin polymerization. Specificity of PCR depends on the specificity of primers and in consequence a pair of primers should comprise some specific properties:

- Typical length of 20-24 bps.
- Absence of dimerization capability.
- Absence of extensive secondary structures (hairpins).
- Lack of secondary binding sites on the template.
- Low binding ability in the 3' end to avoid mispriming.

- Melting temperatures (TM) should be as close as possible between 55oC and 65oC. TM can be calculated as follows: TM= 2 x (A+T) + 4 x (G+C). It is desired that the TM ranges between 55-65 oC. The PCR annealing temperature (TA) should be approximately 5°C lower than the primer melting temperature.

4. Nucleotides (dNTPs): A mixture of activated deoxyribonucleotides (dATP, dTTP, dCTP, dGTP) are the necessary "building blocks" for the synthesis of the DNA strands. It is very important to have equal concentrations of each dNTP as inaccuracy in the concentration of even a single dNTP dramatically increases the misincorporation level.

5. $MgCl_2$: These bivalent ions are necessary for the catalytic action of the polymerase. They chelate with the dNTPs and the nucleotides acquire the proper form for insertion in the growing strand. Also, Mg^{+2} ions regulate the stringency of the interaction between the template and the primers.

6. PCR buffer: Necessary to create the optimal conditions for the activity of the polymerase. For that reason, the buffer contains Tris-HCl which adjusts the pH and monovalent ions of K^+ and Na^+ which in turn regulate the ionic strength.

The amplification of a DNA sequence typically involves 20-30 cycles of PCR and each cycle of PCR requires three temperature shifts. These repeated cycles of temperature shifts are usually done automatically in a thermocycler, a machine that sequentially shifts between the desired temperatures and remains at each temperature for a specified length of time in order for a specific reaction to take place.

Step	Purpose	Temperature / Duration	Number of Cycles
Initial Activation	Thermal activation of the DNA polymerase	94-96 °C / 1-9 min (Or 98°C for extremely stable polymerases)	1
Denature	Denaturing of the DNA template by destroying hydrogen bonds	94-98 °C / 20-30 sec	30
Annealing	Binding of primers on ssDNA	55-65 °C / 20-40 sec	30
Extension	Synthesis of new DNA strands by the DNA polymerase	72 °C / 40 sec	30
Final Extension	To ensure extension of all ssDNA molecules	70-74 °C / 5-15 min	1
Final Hold	"Storage" of the reaction	4 °C / Indefinite	-

The table below shows the ingredients, the concentrations and the amounts used for a typical PCR. All the PCRs performed were adjusted for a final volume of 50 μL. The enzyme Taq Pol as well as the 10X PCR Buffer with $MgCl_2$ included were both purchased from SIGMA-ALDRICH® (Cat.No: D1806 and P2192, respectively). The DNA amount used in each reaction was always 1 μL.

Ingredient	μL in a Vf=50 μL
H_2O	41.5 μL
Taq Polymerase	0.5 μL
10X PCR Buffer	5 μL
Forward primer	0.5 μL
Reverse primer	0.5 μL
100 mM dNTPs	1 μL

The reactions of this experiment were run on a PTC-200 Peltier Thermal Cycler from MJ Research. The PCR program which used, was structured as follows:

- 94°C for 4 min
- 94°C for 45 sec
  55°C for 30 sec $\rbrace$ 34 cycles
  72°C for 1 min
- 72°C for 5 min
- 4°C forever

In every single PCR, a negative as well as a positive control were included in the reaction. The negative control contains all the ingredients of a reaction excluding the DNA and works basically as an indicator of contamination. On the other hand, a positive control refers to a sample which certainly contains the DNA region we wish to amplify. Thus, we can check whether the parameters of the experiment are calibrated correctly.

The primers (SIGMA-ADRICH®) specifically designed for the requirements of the experiment are listed in the table below including their specific characteristics. The table also presents the positive controls which were tested and efficiently worked for each different gene.

The products of the PCRs were visualized using agarose gel electrophoresis.

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GENE	PRIMER	SEQUENCE	T$_M$ °C	T$_A$ °C	MANUFACT.	POS.CONTROL
hαSMA	h-aSMA-F41	CAGCTTCCCTGAACACCACC	65.5	50.5	SIGMA	Fibroblast cDNA
	h-aSMA-R740	ACGCTCAGCAGTAGTAACGA	65.5	50.5	SIGMA	
hSLUG	hSLUG_F_361	ACGCCCAGCTACCCAATGGC	60	55	SIGMA	Viral SLUG
	hSLUG_R_779	AGGCTTCTCCCCCGTGTGAGTT	60	55	SIGMA	
hSNAIL	hSNAIL_F_189	TGCTGGCAGCCATCCCACCT	60	55	SIGMA	pAdTrackCMVSNAIL
	hSNAIL_R_587	GGCAGCGTGTGGCTTCGGAT	60	55	SIGMA	
hTWIST	hTWIST_F_362	5'ACGTGTCCAGCTCGCCAGTC 3'	60	55	SIGMA	pTWIST
	hTWIST_R_881	5'GAGCCGCTCGTGAGCCACAT 3'	60	55	SIGMA	
hVimentin	hVIM_F_821	GCAGCTCAAGGGCCAAGGCA	60	55	SIGMA	HeLa
	hVIM_R_1327	CTGGCGCAGGGCGTCATTGT	60	55	SIGMA	
hN-CADHERIN	hCDH2_F_1137	5'TCGCGAGCAGATAGCCCGGT3'	60	55	SIGMA	293FT
	hCDH2_R_1547	5'GACGGCCGTGGCTGTGTTTG 3'	60	55	SIGMA	
hE-CADHERIN	hCDH1_F_979	AGCCACAGACGCGGACGATG	60	55	SIGMA	MCF7
	hCDH1_R_1523	ACGGTGGCTGTGGAGGTGGT	60	55	SIGMA	
	SIP1_R_485	GGTCCAACAGCCCCGTCAGC	60	55	SIGMA	

2.8 Agarose gel electrophoresis

Gel electrophoresis is an analytical method of separating DNA fragments of different lengths. It is the basic technique for visualizing PCR products. According to the size of the fragments to be separated the gel can be composed of either agarose (used for fragments of 200bp to about 20kb) or polyacrylamide (used for smaller DNA fragments).

The operating principal of the method is that the application of an electric field causes the negatively charged DNA to migrate towards the positive pole of the field. The electric current is the driving force of the separation while the size and the conformation of the nucleic acids are the speed

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determinants of the migration throughout the gel. Long fragments migrate slower (that means shorter) in the gel than short fragments. The electrophoretic mobility of the DNA molecules depends on some basic parameters:

1. Size of the fragments: dsDNA acquire mobility which is reversely correlated to the logarithm of their molecular weight.

2. Conformation of the DNA: supercoiled DNA usually moves faster than relaxed DNA because it is tightly coiled and hence more compact.

3. Electrical current: In principal, the higher the voltage, the faster the DNA moves. The resolution of large DNA fragments however is lower at high voltage.

4. Composition of electrophoresis buffer: In the absence of proper ionic strength the electrical conductance is low and hence the mobility is also affected. High ionic strength can lead to melting of the gel and DNA diffusion.

5. Ethidium Bromide: It intercalates into circular dsDNA affecting the superhelicity of the molecules and also incorporates into linear DNA reducing its mobility.

Agarose gel electrophoresis should consist of some ingredients:

1. Agarose gel: The gel is prepared by dissolving the agarose powder in an appropriate buffer, such as TAE or TBE. The concentration of gel affects the resolution of DNA separation. 1% gels are common for many applications.

2. Buffer: The role of the buffer is to keep stable the distribution of the electrical current throughout the gel. The most common buffers used for nucleic acids are Tris/Acetate/EDTA (TAE) and Tris/Borate/EDTA (TBE). TAE has the lowest buffering capacity but provides the best resolution for larger DNA.

3. Ethidium Bromide (EtBr): Ethidium bromide is an intercalating agent commonly used as a fluorescent tag (nucleic acid stain). UV radiation absorbed by nucleic acids at 260 nm is transferred to the EtBr or it is directly absorbed by EtBr at 302 nm or 366 nm and it is transmitted at 590 nm, in the red-orange area of the visible spectrum.

4. Gel Loading Dye: Contains three basic ingredients:

- Tracking dyes which help to track the progression of gel electrophoresis and sample loading process in the well. The most common tracking dyes are bromophenol blue and xylene cyanol FF. Both tracking dyes carry net negative charge at neutral or slightly basic pH and move like nucleic acids. The bromophenol blue and xylene cyanol co-migrate with ~300 bp and ~4000 bp DNA fragments in 1% agarose gel respectively.

- High density reagent (glycerol, sucrose) is added to increase the density of the sample. Due to high density, sample settles at the bottom of the well.

- Molecular-weight Size Marker which is a set of standards that are used to identify the approximate size of a molecule run on a gel during electrophoresis.

The 1% agarose gels were made of 2g of agarose powder in 200 mL of 1X TAE buffer. After the gel was polymerized it was placed into the running chamber which was filled with 1X TAE buffer. Approximately 300 µL of aqueous solution of EtBr 10mg/ml were poured into the running chamber. 10 µL of gel loading buffer (containing 50% glycerol, 0.01% 0.5X TAE, ddH20, xylene cyanol FF and bromophenol blue) were added into the PCR products. A total of 15 µL was pipetted into the wells and 4 µL of 1Kb DNA Ladder (Promega) were also added. The gel run at 80V for approximately 45 minutes.

The bands were visualized using the fluorescence detector BIORAD ChemiDoc™ MP Imaging System.

2.9 Lysate preparation

To prepare samples for running on a protein separating gel, cells and tissues need to be lysed to release the proteins of interest so that they can migrate through the gel.

The lysis of the previously prepared cell pellets was done as follows: The cell pellet was directly thawed and resuspended into Lysis Buffer P for approximately 10 minutes on ice. The amount of the Lysis Buffer P to be used depends on the size of the pellet. Cell pellets coming from a fully dense T-75 flask require approx. 200 µL. Afterwards, pellets are microcentrifuged at 13.000 g for 10 min. at 4oC. The supernatant (lysate) is carefully transferred into a fresh tube and kept at-20oC for short term storage or can be directly used for SDS-PAGE/Western Blotting. The pellet (cell debris) is discarded.

2.11 Protein quantification assay

The protein quantification of the lysates was based on the Bradford Assay. A BSA (Bovine Serum Albumin) Working solution was prepared by 1:10 dilution out of a 10 mg/ml Stock solution (Roth, #8076.3). The Coomassie Reagent from the Pierce Coomassie Plus™ (Bradford) Assay Kit (Thermo SCIENTIFIC, Prod.#1856210) was diluted as recommended on the bottle.

In a 96 well plate, 50 µL of ddH20 were added in all wells using the pattern above. Working solution of BSA or 1 µL of samples (triplets) were also pipetted according to the scheme. 150 µL of diluted Bradford Reagent were pipetted in the wells. After 5' readout in ELISA reader (Multiskan EX, Thermo Electron Crorporation) at 595 nm, determination of the concentration is done by the software (Ascent Software 2.0) according to the standard curve produced by the different concentrations of the BSA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 Ml	0 µL	0 µL	1 1µL	1µL	1µL						
B	1 µL	1 µL	1 µL	2 1µL	1µL	1µL						
C	2 µL	2 µL	2 µL	3 1µL	1µL	1µL						
D	4 µL	4 µL	4 µL	4 1µL	1µL	1µL						
E	6 µL	6 µL	6 µL		Etc.							
F	8 µL	8 µL	8 µL									
G	10 µL	10 µL	10 µL									
H	12 µL	12 µL	12 µL									
	1mg/ml BSA			SAMPLES								

2.10 SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) and Western Blot

SDS PAGE is a common technique of separating proteins according to their molecular weight after they have been loaded on a polyacrylamide gel.

After determination of protein concentration, samples were diluted 1:1 with equal volume of 2X Laemmli buffer containing 10% β-mercaptoethanol. Samples were heated at 96°C for 10 minutes. The boiled lysates can either be directly loaded for an SDS-PAGE/Western Blot or stored at -20°C till use.

Antibodies typically recognize a small portion of the protein (epitope) and this domain may reside within the three dimensional conformation of the protein. To enable access of the antibody to the epitope it is imperative to unfold the proteins.

The use of a buffer containing an anionic denaturing detergent (SDS) plus the boiling of the lysates are the factors that cause proteins to denature and the epitopes to expose. At the same time, SDS anions, apart from denaturation, confer a negative charge to all proteins. Under these denaturing conditions, the mobility of the proteins is determined entirely by their molecular weight.

The separation of the proteins is dependent on the relative size of the pores within the gels. The total acrylamide is given as a percentage (w/v) of the acrylamide plus the bis-acrylamide which is cross-linking factor. The percentage of the gels will determine the rate of migration and degree of separation.

Protein size (kDa)	Gel Percentage (%)
4-40	20
12-45	15
10-70	12.5
15-100	10
25-200	8

In the present experimental procedure, 40 μg of the lysates were loaded in the wells. Positive control lysates, 40 μg, were also included to demonstrate that the protocol is efficient and correct and that the antibody recognizes the target protein which may not be present in the experimental sample. In case of transduced cells used as positive controls, only 5 μg of the respective lysates were loaded.

Loading Procedure:

After the gel is polymerized it is submerged into the running chamber filled with 1X Running buffer. Samples are loaded along with 5 μL of a protein marker (Protein Marker VI 10 – 245 kDa prestained, AppliChem) that will enable the determination of the protein size and will show the progress of the electrophoretic run. The gel runs at 200V for 45 min and the migration is monitored by the use of the prestained marker.

Blotting Procedure:

An electroblot chamber is assembled by adding inside a cooling unit and a magnetic stir bar. Gel holding cassette is prepared in a specific order: sponge → 2 filter papers → gel → nitrocellulose (NC) membrane → 2 filter papers → sponge. Cassette is closed and inserted into chamber filled with

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1X Transfer buffer. Blotting is done 100V for 1h checking regularly for heating abnormalities.

The principal of the blotting is that, proteins with an electrical charge, conferred by the SDS bound to them, that can be induced to migrate through a gel in an electrical field, can as well be transferred, under an electrical field, from the gel onto a sturdy support, a membrane that "blots" the proteins from the gel. The negatively charged proteins travel towards the positively charged electrode, thus being "captured" by the blotting membrane.

Blocking of the membrane:

Since blotting membranes have an intrinsic ability of binding proteins, measures should be taken for preventing the non-specific binding of primary or secondary antibodies on the membrane. This step eliminates "noise" in the final Western Blot products.

After blotting is complete, the NC membrane is submerged into a 5% milk blocking solution (MBS) for 30 minutes, in room temperature, under mild mechanical agitation.

Incubation with primary antibody:

When the blocking is over, the NC membrane containing the protein of interest is directly incubated with the first antibody which has high affinity and binds to the protein. Typically, a dilution of the 1st antibody is used (in TS-TMBSA) ranging from 1:100-1:3000 depending on the antibody datasheet. The incubation lasted overnight, to ensure specific binding, at 4°C, to avoid contamination and antibody destruction, under gentle agitation.

The list of the antibodies, their characteristics and their dilutions used:

NAME	CODE	SIZE	SPECIES/CLONALITY	DILUTION	MANUFACTURER
Anti-SLUG (L70G2)	9585	30 kDa	rabbit pAb	1:500	Cell Signaling Tech.
Anti-SNAIL	3895	29 kDa	mouse mAb	1:1000	Cell Signaling Tech.
Anti-TWIST (L21)	134136	28 kDa	rabbit pAb	1:200	SANTA CRUZ BIOTECHNOLOGY, INC
Anti-Vimentin (5GEF10)	3390	57 kDa	mouse mAb	1:1000	Cell Signaling Tech.
Anti-ECadherin	4065	135 kDa	rabbit pAb	1:1000	Cell Signaling Tech.

After incubation, 3 short washings with 20X TBST were performed to remove any residual primary antibody.

Incubation with secondary antibody:

The secondary antibody is raised against the host species used to generate the primary antibody. The secondary antibody has to be directed against the isotype of the primary antibody.

Secondary antibodies were diluted in TBST based on the datasheet. Usually a range of dilution from 1:3000-1:20.000 is applied. The membrane was incubated with 7 mL of 5% MBS, 14 μL of TBST and 7 μL of secondary antibody conjugated with HRP (1:3000) for 1h at RT under gentle shaking.

Afterwards, 1 short and two 10 minutes lasting washes of the membrane with 20X TBST were performed to remove unbound antibody.

NAME	#	DILUTION	MANUFACTURER
Goat anti-mouse IgG-HRP	SC 2005	1:3000	SANTA CRUZ BIOTECHNOLOGY, INC
Goat anti-rabbit IgG-HRP	SC 2030	1:3000	SANTA CRUZ BIOTECHNOLOGY, INC

Development of membrane-bound protein:

The detection was done using the Clarity™ Western ECL Substrate Kit (BIO-RAD), which is compatible with HRP-conjugated secondary antibodies, according to manufacturer's instructions. The enzyme catalyzes the oxidation of Luminol in the presence of a peroxide and leads to the formulation of 3-aminophthalate dianion and the release of light. The substrate kit components are mixed in 1:1 ratio. For a mini-sized membrane (7 x 8.5 cm2) 7 mL of solution are adequate. The intensity of the signal depends on the amount of the secondary antibody bound and thus is directly proportional to the original amount of the protein in the sample. Membrane was incubated with the solution for 2 minutes and the signal was produced using the BIO-RAD ChemiDoc™ MP Imaging System and the respective software.

GAPDH (Loading Control) Probing

Like in any other experimental procedure, in Western Blot, the use of a loading control is essential. The loading control ensures that the same amount of protein is loaded in each lane, proteins from different lanes are transferred with equal efficiency from the gel onto the membrane and that antibody incubation (primary or secondary) and detection method are consistent across different lanes.

For whole cell lysates, as in the specific experiment, GAPDH was chosen as a loading control since it is a housekeeping protein and expressed in all tissues.

The protocol followed for GAPDH probing is exactly the same with the one described above.

3. RESULTS

3.1. Immortalized HBVPs

10^6 HBVPs were successfully immortalized using $1x10^7$ infection particles of the SV40 Large T-antigen lentiviral construct. Cells emitted red light because of RFP expression, when checked under the fluorescence microscope. Cell selection was performed using 1 μg/ml of puromycin. Of the total number of cells that survived immortalization, 30% showed RFP expression. Based on the level of fluorescence, which in turn indicates the number of the SV40-RFP-PURO lentiviral constructs integrated into the cellular genome, HBVPs were divided into two groups: One with a high level of fluorescence (HIGH population) and one with a low level of fluorescence (LOW population). Primary HBVPs were expanded in culture for approximately passage number 10-12. After that point they ceased to proliferate. In contrast, HIGH and LOW populations were expanded in vitro, for passage number >18 and so have been shown to be immortalized.
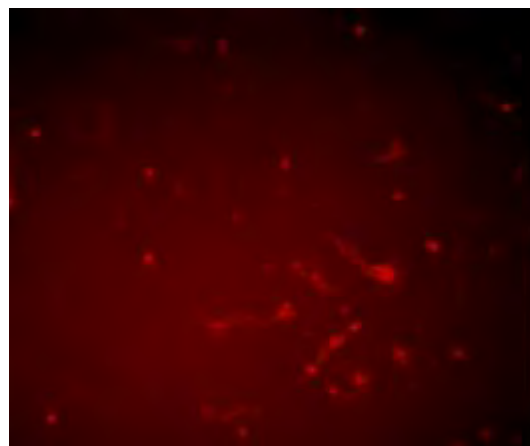
Fig.A: Lenti-SV40-RFP-PURO immortalised HBVPs ($1x10^7$ IFU). Cells were selected with puromycin (1μg/ml) for 1 week and surviving cells were observed with fluorescence microscopy (5X magnification).

3.2 Control PCRs

HBVP samples (primary p6, HIGH Lenti-SV40-GFP-PURO HBVPs p15, LOW Lenti-SV40-GFP-PURO HBVPs p16) were tested using RT-PCR, for the expression of β-actin which serves as a control for the quality of the isolated mRNA and the efficiency of the cDNA synthesis. All samples express β-actin equally, indicating good quality of extracted mRNA and successful cDNA synthesis.

Fig.B: β-actin specific RT-PCR for primary HBVP p6, HIGH Lenti-SV40-GFP-PURO HBVPs p15 and LOW Lenti-SV40-GFP-PURO HBVPs p16;(-)CNT:water;(+)CNT: PCR of the β-actin coding plasmid pGEM- βAct.

To demonstrate the expression of a pericyte specific marker, RT-PCR was performed for α-SMA. α-SMA expression was observed in all samples and human fibroblast cDNA was used as a positive control.

Fig.C: RT-PCR for α-SMA expression in primary HBVP p6, HIGH Lenti-SV40-GFP-PURO HBVPs p15 and LOW Lenti-SV40-GFP-PURO HBVPs p16;(-)CNT:water;(+)CNT: human fibroblast cDNA.
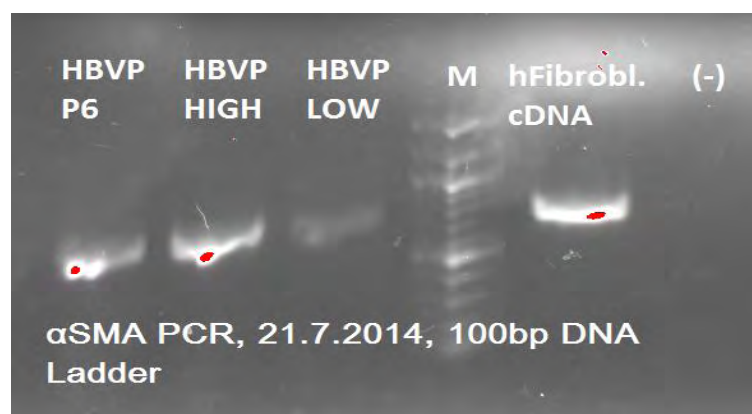
3.2 PCR testing of pericyte specific markers

All three of the HBVP samples (primary p6, HIGH Lenti-SV40-GFP-PURO HBVPs p15, LOW Lenti-SV40-GFP-PURO HBVPs p16) were tested for the expression of three HBVP specific markers: huNG2/CSPG4, huPDGFRβ, CD146/MCAM/MUC18. PCR results for each gene are shown below. All samples were positive for the expression of the markers. The same the pericytes were used as positive controls.
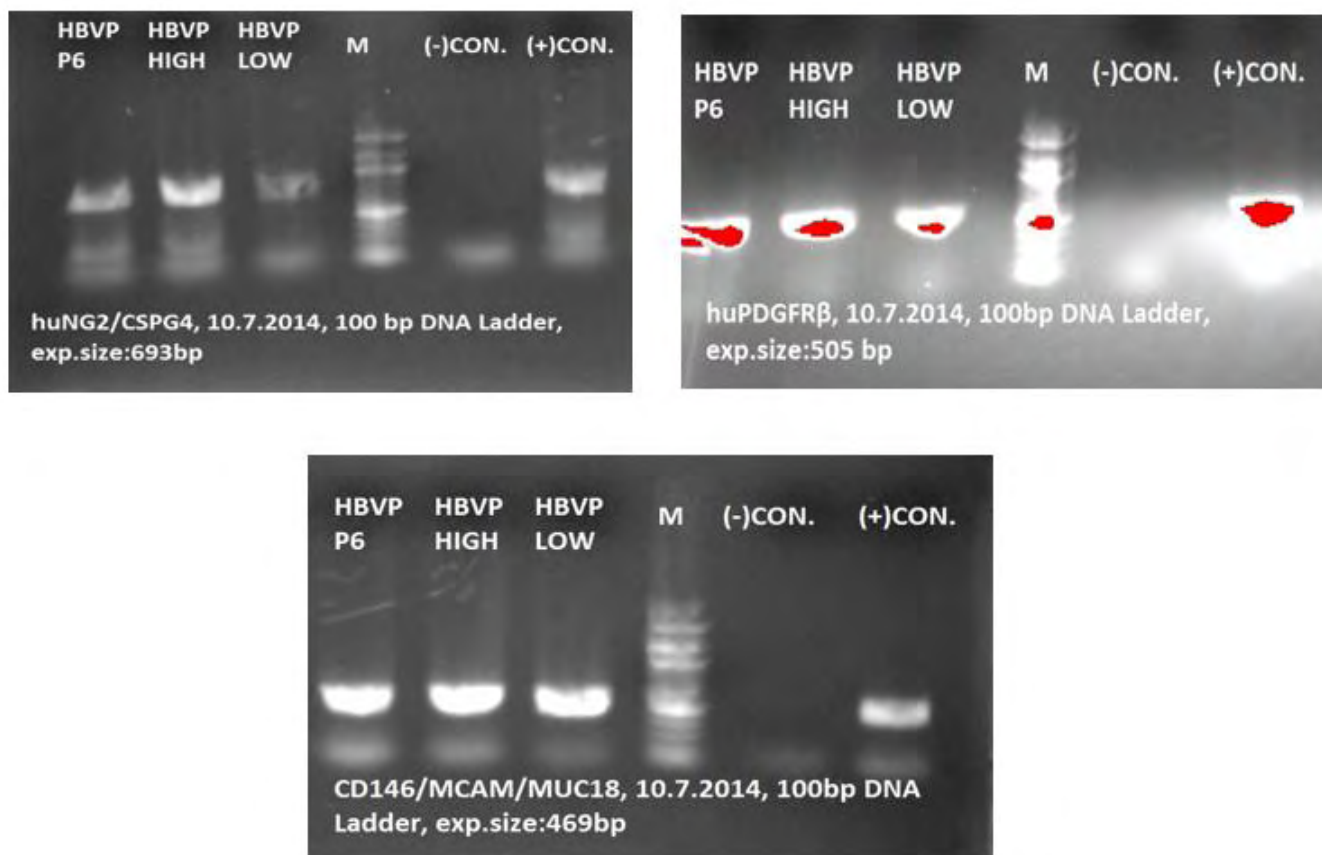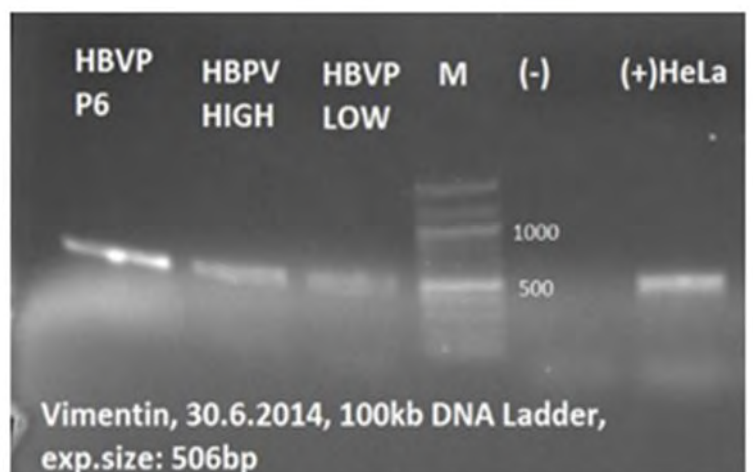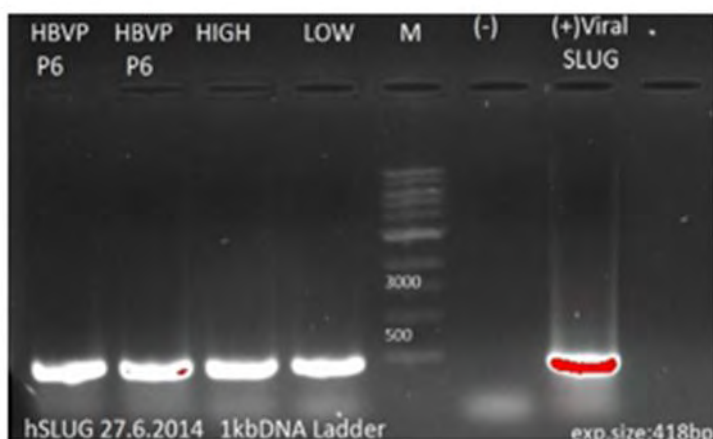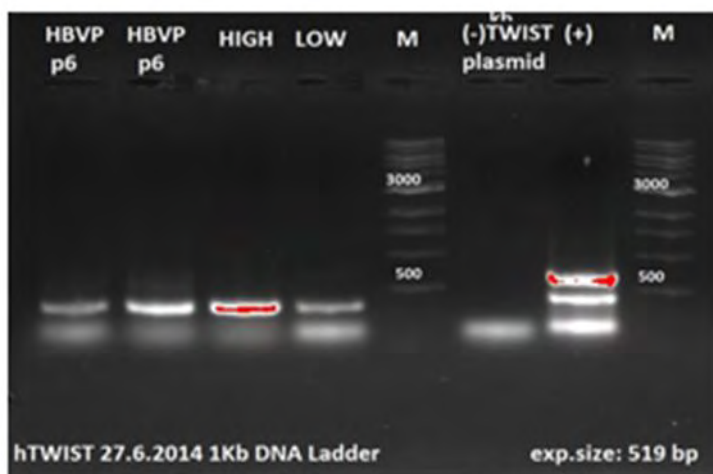

Fig.D: RT-PCR for huNG2/huPDGFRβ/CD146 pericyte specific markers expression in primary HBVP p6, HIGH Lenti-SV40-GFP-PURO HBVPs p15 and LOW Lenti-SV40-GFP-PURO HBVPs p16;(-)CNT:water;(+)CNT: HBVP cDNA.

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3.3 PCR testing of study genes: Slug, Snail, Twist, E-Cadherin, N-Cadherin, Vimentin

All three of the HBVP samples (primary p6, HIGH Lenti-SV40-GFP-PURO HBVPs p15, LOW Lenti-SV40-GFP-PURO HBVPs p16) were tested for the expression of Slug, Snail, Twist, E-Cadherin, N-Cadherin, and Vimentin. All samples were positive for Slug, Snail, Vimentin and N-Cadherin expression but neither Twist nor E-Cadherin were detected. The positive controls used for each gene were: For Slug→Adeno-Slug infected LNT229 GBM cells, for Snail→pAd-TRACK-CMV-Snail, for Twist→pcDNA3-Twist, for Vimentin→Hela cells cDNA, for E-Cadherin→MCF7 cells cDNA and for N-Cadherin→293FT cells cDNA.
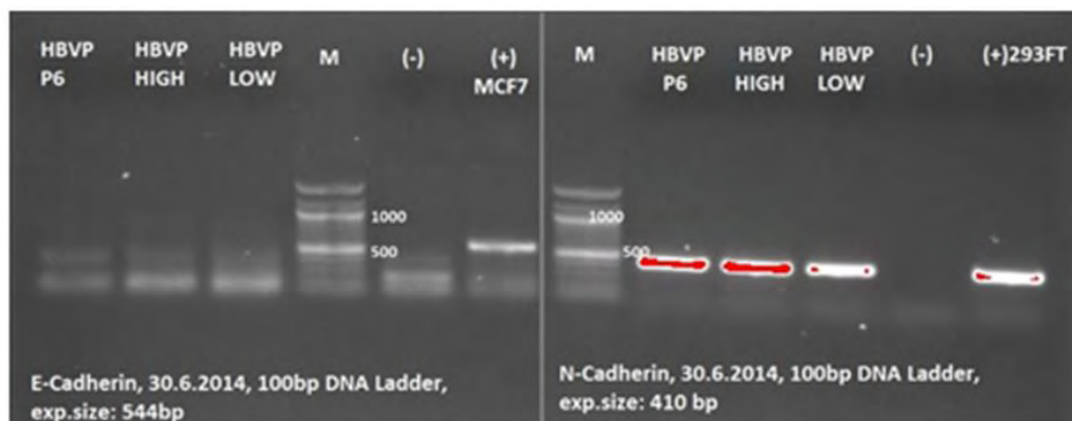
Fig.E: RT-PCR Slug, Snail, Twist, Vimentin, E-Cadherin and N-Cadherin pericyte specific in primary HBVP p6, HIGH Lenti-SV40-GFP-PURO HBVPs p15 and LOW Lenti-SV40-GFP-PURO HBVPs p16;(-)CNT:water;(+)CNT: Slug→ Adeno-Slug infected LNT229 GBM cells, Snail→ pAd-TRACK-CMV-Snail, Twist→ pcDNA3-Twist, Vimentin→ Hela cells cDNA, E-Cadherin→ MCF7 cells cDNA, N-Cadherin→ 293FT cells cDNA.

3.4 Western Blots for E-Cadherin, N-Cadherin, Vimentin

HBVPs cell lysates (primary p6, HIGH Lenti-SV40-GFP-PURO HBVPs p15, LOW Lenti-SV40-GFP-PURO HBVPs p16) were analyzed for EMT protein markers using immunoblot assays for E-Cadherin (135 kDa), Vimentin (57 kDa), Slug (30 kDa), Snail (29 kDa) and Twist (28 kDa). MCF-7 lysate was used as a positive control for E-Cadherin and as a negative control for Vimentin. LNT229 GBM adenoviral infected cells overexpressing Slug (229AdSlug), Snail (229AdSnail) and Twist (229AdTwist) were used as positive controls for the three proteins. All blots were reprobed for GAPDH (30-40 kDa) which functions as a loading control. All bands corresponded to their expected molecular size band in the marker. Very low amounts of Snail and high amounts of Vimentin were detected at a protein level in all samples. In line with RT-PCR results, absence of E-Cadherin and Twist was confirmed. Interestingly, Slug mRNA was detected in all samples whereas Slug protein was not detected with the immunoblot assay.
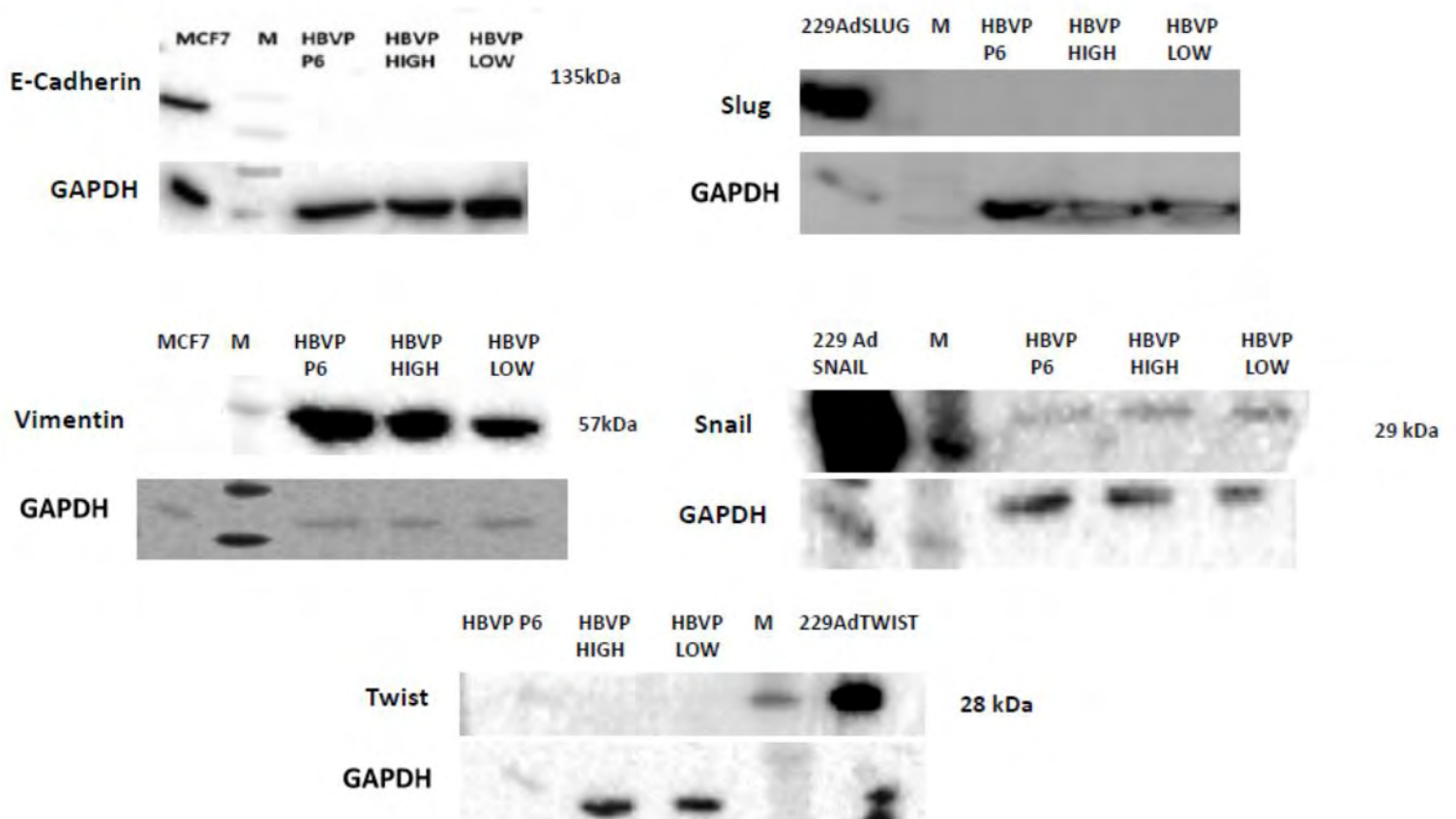
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Fig.F: Immunoblot for Slug, Snail, Twist, Vimentin and E-Cadherin in primary HBVP p6, HIGH Lenti-SV40-GFP-PURO HBVPs p15 and LOW Lenti-SV40-GFP-PURO HBVPs p16 lysates;(+)CNT: Slug→ Adeno-Slug infected LNT229 GBM cells lysate, Snail→ Adeno-Snail infected LNT229 GBM cells lysate, Twist→ Adeno-Twist LNT229 GBM cells lysate, E-Cadherin→ MCF7 cells lysate; (-)CNT: Vimentin→MCF7 cells lysate.

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4. DISCUSSION

The purpose of this study was to generate an immortalized cell line of human brain vascular pericytes and compare the expression profiles of pericyte specific factors and critical EMT involved factors between primary and immortalized Lenti-SV40-RFP-PURO HBVPs.

The immortalization of the cells was successfully achieved using the large T-antigen of the SV40 Simian virus. Lots of studies have shown that immortalization via SV40 large T antigen, is an efficient approach for the generation of long-term cell cultures and that the process is basically non-tumorigenic allowing cells to maintain their primary phenotype (Li et al. 2013, Lehmann et al. 2012, Huang et al. 2012). SV40 infection interferes with many critical pathways related to cell cycle control and DNA repair. The T-antigen binds to and inhibits the cell cycle guardians, tumor suppressors p53 and pRB. Furthermore the SV40 infection causes IGF2 (insulin-like growth factor) secretion and upregulation of the IGF2 receptor, promoting cell growth (Foddis et al. 2002).

To begin with, parental as well as immortalized pericytes were tested for a series of well characterized markers which are not exclusively found in pericytes, but are used for their identification. Desmin and α-smooth muscle actin (α-SMA) are contractile filaments and their location is intracellular. Since pericytes belong to the same lineage and category of cells as vSMCs they are expected to produce α-SMA. α-SMA has been demonstrated in cytoplasmic microfilament bundles of pericytes (Diaz-Flores et al. 1991). Neuron-glial 2 (NG2) or high molecular weight melanoma-associated antigen (HMWMAA) is a chondroitin sulfate proteoglycan, whose expression is restricted to arteriolar and capillary

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perivascular cells (vSMC and pericytes). Platelet-derived growth factor receptor (PDGFRβ) is a tyrosine-kinase receptor and the most widely use pericyte marker. PDGFRβ has been implicated as an important receptor for recruitment and survival of pericytes by paracrine secretion of PDGFB by endothelial cells (Hall 2006, Armulik et al. 2011).

Crisan and his colleagues, by publishing a landmark paper, managed to validate the CD146+ NG2+ PDGFRβ+ ALP+ CD34– CD45– vWF– CD144– phenotype as an indicator of pericyte/perivascular cell identity throughout human fetal and adult organs and at the same time observed that each individual perivascular cell has multilineage mesodermal potential and therefore adheres to the strict definition of a mesenchymal stem cell (Crisan et al 2008). Thus, intriguing links between MSCs and vascular pericytes have been established. In the present experimental process, early parental (p6) as well as immortalized pericytes (HIGH p15 and LOW p16) were positive for CD146+/NG2+/PDGFRβ+ and αSMA+.

After the identity of the pericytes was confirmed, the purpose was to examine the expression of the EMT factors Slug, Snail, Twist in both primary and Lenti-SV40-RFP-PURO immortalized pericytes. These factors should not be present in pericytes recruited in normal brain vasculature in contrast to GBM associated pericytes where the expression of at least Slug and Twist has been verified (unpublished results). The EMT program, apart from its role in normal developmental processes and wound healing, has been proposed as a major contributor to tumor progression and invasion. Only quite recently a few studies have ascribed a potential role of EMT factors Snail and Twist as putative promoters of GBM invasion (Mikheeva et al. 2010, Myung et al. 2014). Moreover, among the several

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molecular subtypes of GBM that have been identified because of their differential gene expression patterns, the mesenchymal subtype showed high expression of EMT-related genes and poor prognosis (Phillips et al. 2006). Both parental and immortalised HBVPs (HIGH p15 and LOW p16) express Slug and Snail mRNA but do not express Twist mRNA. The immunoblot assays revealed that only Snail can be found at a very low protein level while Slug and Twist are not detected. The discrepancy between Snail mRNA and Snail protein could be due to differential translation of Snail mRNA or due to post-translational mechanism modification which leads to fast degradation of the protein. Respectively, absence of Twist protein can be attributed to post-translational silencing of Twist mRNA or fast post-translational degradation of Twist protein.

The results above indicate that immortalised HBVPs can be distinguished from GBM associated pericytes which are shown to express at least Slug and Twist. At the same time, the successful immortalization of the HBVPs renders them a workable cell line for in vitro studies. In consequence, they can be used to study the effects of vessel associated GBM cells or GBM-derived factors, such as TGFβ, on the expression of EMT factors in pericytes. It would be also useful to determine the effects of EMT overexpression on the differentiation and/or functional characteristics of the pericytes.

Trevino-Villarreal, and his colleagues, revealed also a possible role of pericytes in EMT initiation in the context of melanoma progression. More specifically, they showed that pericyte production of TGF-β promotes B16 melanoma cell development of EMT, as shown by loss of E-cadherin expression and up-regulation of the mesenchymal markers N-cadherin

and vimentin, resulting in an increase in melanoma cell tumorigenicity (Trevino-Villarreal et al. 2012). This mechanism could work inversely in the case of glioblastoma. Given that GBM cells secrete big amounts of TGFβ due to their immunosuppressive nature, (Leitlein et al. 2001) we could assume that TGFβ, acts on closely located pericytes and drives the expression of EMT factors. However, this scenario needs further investigation.

Both primary HBVPs and immortalized HBVPs (HIGH p15 and LOW p16) express N-Cadherin mRNA and Vimentin mRNA while a lack of E-Cadherin mRNA is noticed. Presence of Vimentin and absence of E-Cadherin were also confirmed in protein level. N-Cadherin, is a basic component of adherence junctions located to peg-socket contacts between endothelial cells and pericytes. These junction complexes support transmission of mechanical contractile forces from the pericytes to the endothelium and apart from N-Cadherin, they also contain, cell-adhesion molecules, β-catenin based adherens junctions and ECM components like fibronectin (Armulik et al. 2005). Both E-cadherin and N-cadherin belong to classical cadherins. Although they mediate cell-cell adhesion through the same mechanism, their actual effects are completely contradictory. Loss of E-cadherin is usually associated with the appearance of N-cadherin in a phenomenon referred as "the cadherin switch." Researchers suggest that E-cadherin could switch to N-cadherin to result in an increase of N-cadherin and a decrease of E-cadherin (Rai et al. 2014). N-cadherin plays a more critical role in inducing tumor infiltration and metastasis than E-cadherin. That is why EMT program, driven by Slug, Snail and Twist facilitates primarily suppression of the E-Cadherin gene and upregulation of N-Cadherin (Zavadil et al. 2005). Since in our cells samples low levels of

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Snail protein have been detected we could assume that absence of E-Cadherin mRNA and protein as well as detection of N-Cadherin mRNA might be due to Snail which is known to repress E-Cadherin expression. However, N-Cadherin immunoblot assay was not performed and we cannot conclude for sure. As far as vimentin is concerned, its expression in pericyte was anticipated. Electron microscopy studies have revealed a network of cytoplasmic filaments. Pericytic intermediate filament proteins show species and tissue differences expressing vimentin or vimentin and desmin (Diaz-Flores et al. 1991).

Perspectives

On the whole, we managed to generate an immortalized human brain vascular pericyte cell line using lentiviral vectors who carried the SV40 large T-antigen. It would be useful to see, in the long run, whether the effect of the SV40 large T-antigen on the brain vascular pericytes caused actual immortalization or simply an extension of their proliferative capacity. Generation of such cell lines renders them a useful tool for continuous in vitro studies. The challenges of defining a pericyte have not been made easier due to the fact that no general pan-pericyte molecule has been found. And that is because of their multifaceted functions, unknown origins and the various locations found within the human body. However, some dynamic molecular factors- NG2, PDGFRβ, CD146, α-SMA serve as markers enough for the identification of pericytes. The presence of the markers in both parental and immortalised HBVPs indicates that the immortalization process did not cause any phenotypic change, at least short term. HBVPs of this study were also found to express the EMT specific protein marker Snail even at low levels. Given their in vivo

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localization in perivascular niches of GBMs as well as the recently upcoming evidences of EMT molecular signatures in glioblastomas, which account for tumor invasiveness, perspective studies should shed light on the putative role of GBM cells, GBM secreted TGF-β and pericytes in EMT initiation. And if any sound evidences of pericyte contribution in EMT are educed, then alternative molecular therapeutic approaches could be designed for the disruption of this relationship and the mitigation of tumor invasiveness and metastasis.

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