수의학석사 학위논문

Development of angiogenesis assays using 3D-cell spheres

3 차원 구체를 이용한 신생 혈관 형성 분석법 연구

2013 년 2 월

서울대학교 대학원

수의학과 인수공통동물질병학 전공

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이 논문을 수의학석사 학위논문으로 제출함
2013 년 2 월

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오수전의 수의학석사 학위논문을 인준함
2013 년 2 월

위 원 장 강 경 선 (인)

부위원장 Andreas Kurtz (인)

위 원 박 세 창 (인)

A dissertation for the Degree of Master

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February 2013

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ABSTRACT

Development of angiogenesis assays using 3D-cell spheres

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3-dimensional (3D) cell culture systems are superior over conventional 2D-systems as they more naturally model the in vivo situation in terms of cell-cell and cell- matrix interactions. 3D-models are therefore preferable for in vitro drug screening and the analysis of biological processes that are relevant e.g. for tumor angiogenesis. A disadvantage

of 3D-systems is the lack of standardized high throughput and high content screening (HCS) technologies.

The most established in vitro 3D system is the cultivation of the cells in suspension in order to promote the establishment of spheres. Here, we aimed to investigate whether a cell-sphere based pipeline for the analysis of neoangiogenesis by HCS technology can be developed. HCS is an optical method allowing high-throughput analysis of cells.

We established spheres from neurofibrosarcoma, mesenchymal and human induced pluripotent stem cells (embryoid bodies, EB) for the analysis of the tumor-driven induction of endothelial markers in the stem cell spheres as a model for neoangiogenesis. To this end tumor spheres and stem cell spheres were contact-co-cultured. This model was then tested for applicability in HCS and it was shown that the early endothelial marker vascular endothelial growth factor receptor 2 (VEGFR2) is induced by tumor spheres in EBs. Furthermore, to enable reproducible HCS of sphere co-cultures, a matrix-printing method for selective and spatially controlled attachment of spheres was established and tested.

In conclusion, the work (i) established a sphere-based 3D technology to analyze angiogenic induction by tumor cells and (ii) developed a matrix printed array that facilitates arrangement of spheres and controlling of confrontation cultures for high content screening.

Keywords : 3D cell culture, neurofibrosarcoma, high content screening

Student number : 2011-21699

LIST OF ABBREVIATION

2D	2 dimension
3D	3 dimension
MPNST	Malignant peripheral nerve sheath tumor
PDMS	Polydimethylsiloxane
ES	Embryonic stem cell
EB	Embroid body
MSC	Mesenchymal stem cell
HUVEC	Human umbilical vein endothelial cell
hiPSC	Human induced pluripotent stem cell
CD49b	Integrin alpha 2
CD271	Nerve growth factor receptor
VEGF R	Vascular endothelial growth factor receptor

VCAM	Vascular cell adhesion molecule
GeSiM	Gesellschaft fuer Silizium-Mikrosysteme mbH
HCS	High content screener

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INTRODUCTION

Gene expression profiles of three-dimensional (3D) cell cultures have been shown to reflect more accurately the clinical in vivo situation than those observed in 2D cultures. 3D - culture models can be used to evaluate therapeutic methods and offer the promise of improving clinical efficacy predictions. Indeed, the potential of sophisticated, 3D - culture systems to be incorporated into mainstream development processes for new anti-cancer therapeutic strategies is increasingly recognized. Among the 3D in vitro culture systems, which have been established to restore the histomorphological, functional and microenvironmental features of in vivo human tissue are tumor spheroid or organ specific sphere cultures such as neurospheres and nephrospheres. Tumor spheres present a classical approache to obtain and maintain the functional phenotype of human tumor cells and they considerably contributed to our knowledge of cellular responses to

diverse therapeutic interventions. However, the use of advanced 3D spheroid models would receive greater interest if high-throughput manipulation and analysis methods became available.

It is commonly accepted that tumor angiogenesis is a prerequisite for tumor growth[1]. Angiogenesis is stimulated by angiogenic factors released by tumor cells, and by cell-cell and cell-matrix interactions[2,3]. Although several in vitro 2D-angiogenesis and semi-3D models exist, the determination of anti-angiogenic efficacy of potential drugs is inaccurate and requires a 3D-environment with the possibility for proper cell-cell and cell-matrix interactions. Animal models of angiogenesis are also commonly used, but lack the species specificity of the human body. Hence, to determine angiogenesis and anti-angiogenic drug efficacies, the use of a full in vitro 3D-model with human cells would be of great benefit, especially if this model also allows for high-content, high throughput screening.

Tumors are angioneic and induce vasularization and endothelialization of their stroma. In fact, tumor stem cells have been shown to transdifferentiate into endothelial cells and thus contribute to

the tumor vasculature[4]. The angiogenic properties of tumors can be measured in vitro by providing a reactive cell that is an endothelial cell or is able to differentiate into endothelial cells, reiterating the processes of vasculogenesis and neo-angiogenesis that happen during tumor growth. This reactive cell type to determine neo-angiogenesis can be pluripotent stem cells (PSC), or also mesenchymal stem cells (MSC). For both, endothelial differentiation has been shown[5,6]. In addition, the ability of MSCs to self-renew and to differentiate makes them a promising avenue for clinical applications in regenerative medicine[7]. MSCs localize to sites of hematopoiesis, sites of inflammation, and sites of injury as well as to solid tumors, and are usually found associated to the vasculature. Closely resembling pericytes, MSC have also been shown to undergo endothelial transition. The availability of 3D-tumor spheres and 3D - spheres of reactive cells (PSC, MSC) may thus offer the opportunity to develop an efficient in vitro system for measuring neo-angiogenesis and subsequent vasculogenesis, which is suitable for high content and high throughput assays.

In this research, we were focused on phenotypic changes of 3D -

tumor and stem cell spheroids, and developed technology for their analysis in high content screening systems.

MATERIALS AND METHODS

Cells

The cells which were used in this research can be categorized as pluripotent, multipotent and tumor cells. Human mesenchymal stem cells (MSC) were isolated from human adipose tissue and applied between passages 4 and 6. An induced human pluripotent stem cell (hiPSC) line, derived from cord blood, was used (hCBiPSC3, kindly provided by Hans Schöler, Münster). The neurofibrosarcoma derived cell line S462 was used as a highly angiogenic malignant tumor model. S462 was transfected with a GFP-gene under the control of the CMV promotor. S462 and MSC were grown in DMEM/low glucose (Gibco) containing 10% FBS (Fetal Bovine Serum, Invitrogen), hiPSC were cultivated feeder-free in mTeSR medium (Invitrogen). Human umbilical cord endothelial cells (HUVEC) were cultivated in EGM2 medium (Gibco).

Sphere formation

Tumorspheres, MSC-spheres and embryoid bodies from hCBiPSC3 were formed by using the hanging drop method (Figure 1). This method is highly reproducible and appropriate for making same size spheroids. Spheres of 350~400μm (approximately 3000 cells) were generated. Cells were prepared by trypsinization (Biochrom), washed in DMEM/10%FCS and distributed in 15μl drops with their cell-specific cultivation medium without growth factor supplements.

Angiogenesis assays

Confrontation culture: In confrontation cultures, two spheres are annealed to each other ('confronted') to enable cell-cell interaction. For the generation of confrontation cultures, spheroids were collected and transferred to a 96 well Ultra-Low attachment plate, round bottom (Corning). Two spheres were placed in each well and sedimented to anneal to each other to form confrontation cultures. Tumor spheroids and MSC-spheres were collected at day 3-4, embryonic bodies at day 2-3. EB medium (Invitrogen) was used for every well and changed every

day.

Development of High-Throughput 3D assay for High content screening

For high content screening, spheres, or confrontation cultures, need to be fixed at a predetermined spot for accurate and standardized measurements. To develop means for sphere attachment, we printed a matrix spot on a low attachment plastic surface. The preformed spheres should only attach at the printed matrix spots. To test this approach, we printed Matrigel (Invitrogen) as matrix (Figure 2). Ultra low attachment plates (Corning) were used and Matrigel printed at 4 0C for at different spot diameters and distances.

Immunohistochemistry

Cells were fixed in histofix (paraformaldehyde, Roth) for 30 minutes. For washing and blocking, 10% Glycine solution was used after that for 30 minutes. 10% Donkey serum (Invitrogen) was used for blocking. For permeabilization, 0.01% Triton X-100(Sigma) in PBS was used.

For primary antibodies, we used monoclonal anti-human integrin alpha 2 (CD49b) (R&D systems), polyclonal rabbit anti-human VCAM-1 (R&D systems), monoclonal anti-human VEGF R2 (R&D systems), monoclonal anti-human CD271 (Abcam). After washing in PBS/BSA, cells were incubated with fluorescence-labeled secondary antibodies (figure legends).

Imaging

We used high content screener (HCS) (Operetta, Perkin Elmer) to image the fluorescence recordings. Live cell imaging was performed daily with HCS. 3D images of spheres were reconstructed by stacking image planes.

RESULTS

Adhesion experiments with mesenchymal stem cells and induced pluripotent stem cells

Recent data suggested that micro-contact printing on regular cell adhesion plates followed by pluronic inactivation allows directed embryonic stem (ES) cell adhesion. The principle states that matrigel coated PDMS stamps enable printing of the PDMS stamp-pattern onto a cell culture dish. Under serum-free conditions, cells specifically attached to these patterned regions. Here, matrigel was initially spotted directly onto different cell culture plates to investigate whether cell attachment can be controlled. The findings indicate that MSC specifically attach only to the matrigel spots (Figure 3A-C). To test whether spheres also attach to the matrigel spots, EBs were used. These spheres also attached specifically to the matrigel spots (Figure 3). The main drawback was however, that manually added spots had a diameter

of at least 500 µm (Figure 3D), which is significantly larger than EBs or MSC spheres, which had a size of around 100-300 µm. EB attachment therefore lead to eventual migration and spreading of the cells after 1 week, destructing the spherical properties of the EBs (Figure 3F).

Co-operation with GeSiM for µ-contact printing of matrigel

After I showed that 3D-spheres specifically attach and remain stable for at least 1 week, we attempted to arrange spots for confrontation cultures. Spot size and distance was calculated for optimal arrangement and later usage for high content screening. Printing was performed by the GeSiM (Gesellschaft fuer Silizium-Mikrosysteme mbH). Several tests were conducted using different robot tips to determine the amount of “drops” required to achieve desired spot sizes. Pictures were taken from each well to show spotting arrangement (Figure 4).

Confrontation culture analysis

Confrontation culture was performed with Embryonic bodies (EBs) from hiPSC, MSC and tumor spheres from S462-GFP neurofibrosarcoma cells (Figure 5). Spheres were annealed for confrontation and monitored for 5 days. The spheres confront and fuse into one structure after 3 days. However, cell identities can still be distinguished.

By immunohistochemistry analysis of the embryonic body and tumor sphere confrontation, we could see that the expression of human integrin alpha (CD49b) increased in the EB, while expression of CD271 (p75NGFR) remains contained in the S462-GFP tumor sphere (Figure 6 upper part).

Analysis for endothelial markers (VEGF R2, VCAM) showed an increase with time in the EB (Figure 6, lower part). The most likely explanation is that the tumor spheres induced an endothelial phenotype

in the EBs. In addition, we observed a migration of GFP-positive S462 cells into the and around the EBs. Whether this finding associates with the malignant potency of the tumor cells or is a general feature of tumor cells remains to be elucidated.

The observed effects in EB – S462-GFP tumor sphere confrontations were not observed in control EB-EB and S462-S462 confrontations (Figures 7 and 8).

We have furthermore quantified changes in the expression of endothelial markers based on immunohistochemistry. Spheres from S462 tumor cells, from MSC and from MSC/HUVEC cells were co-cultured and the number of spheres in which the respective markers were detectable was counted. The results show that MSC and MSC-HUVEC spheres increase the number of S462-spheres expressing VEGF R2 and VCAM by 100% (Table 1). No change in expression was observed in MSC or MSC/HUVEC spheres. No changes were found for CD271 and CD49b.

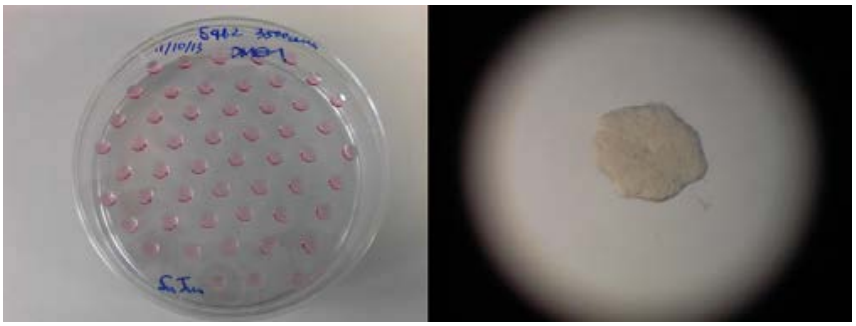

Figure 1. Hanging drop method

Left: Hanging drop arrangement; Right: Tumor sphere after 4 days of cultivation in a hanging drop.

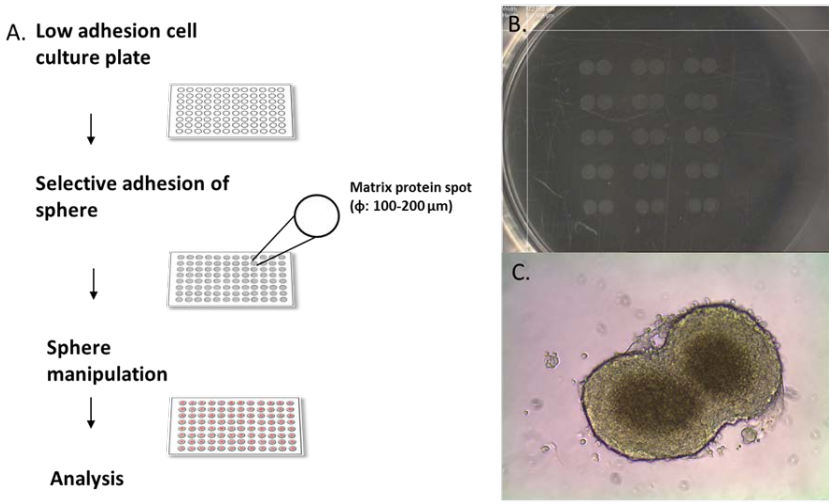

Figure 2. Spotting technique for confrontation

A: Making plates for sphere. B: Matrigel spots for confrontation culture.

C: Confronted sphere on the Matrigel spots.

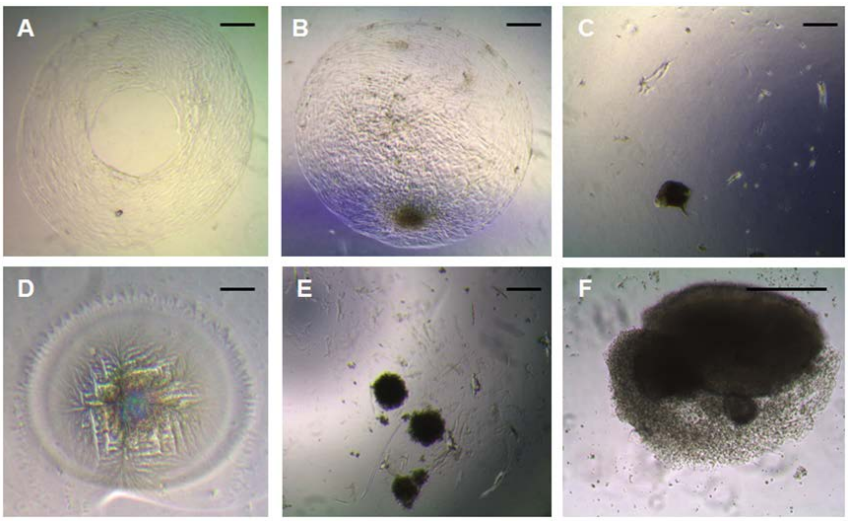

Figure 3. Cell adhesion to matrigel spots.

MSCs were seeded as single cells onto wells of an ultra-low attachment plate with matrigel spots. Change in cell growth was recorded on day 2 (A), 6 (B) and 9 (C) after seeding. (D) Matrigel spot 1 hour after addition of 0.5 µl undiluted matrigel. 3-7 day old EBs were added to a matrigel-spotted well of an ultra-low attachment plate and the change in cell growth recorded three days (E) and 1 week (F) after EB addition. Scale bar represents 100 µm.

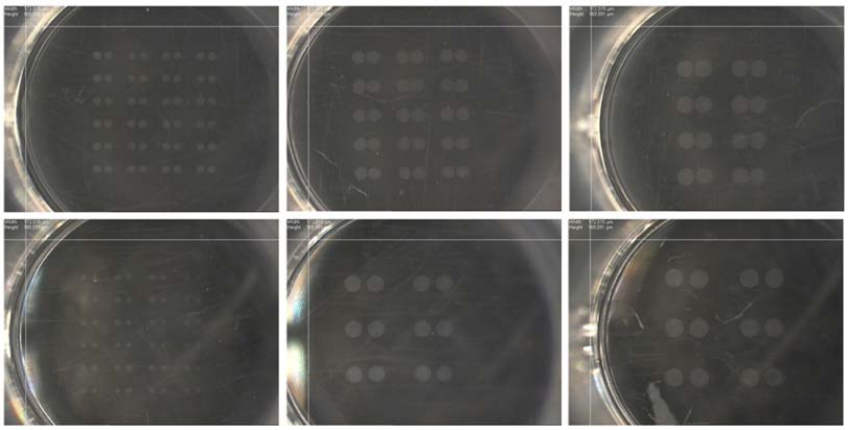

Figure 4. Representatives of different spot sizes and distances.

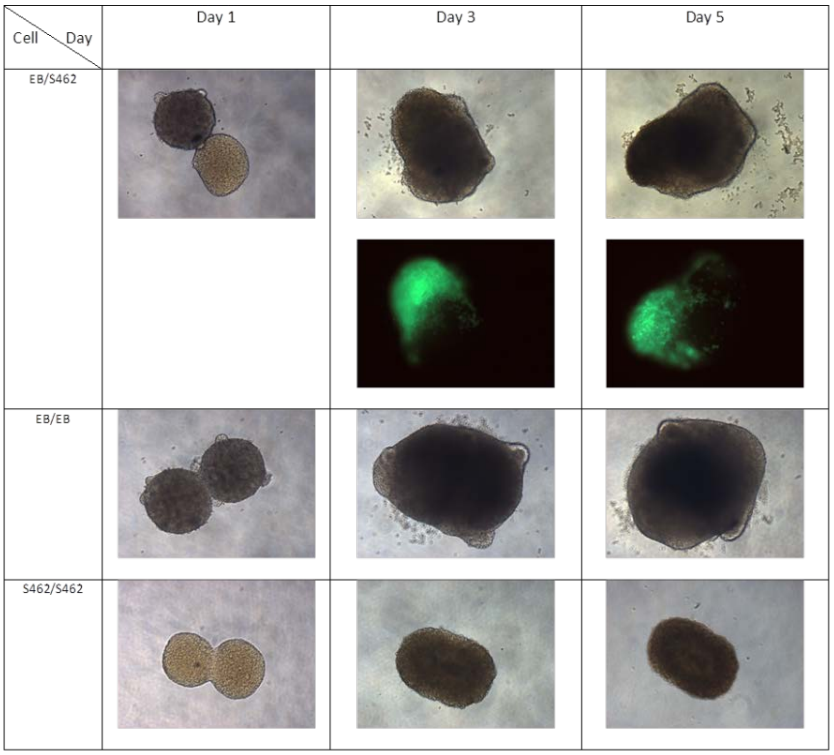

Figure 5. Confrontation cultures at day 1, 3 and 5; brightfield images.

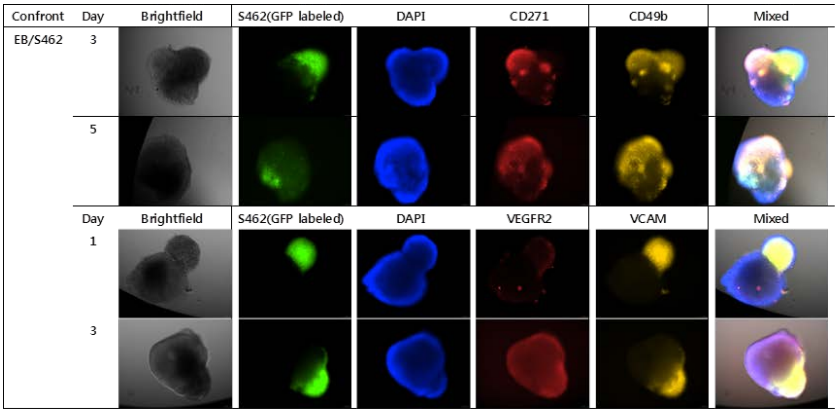

Figure 6. Immunostaining of EB and tumor confrontation at different time point in high content screener for cell adhesion and endothelial markers.

Upper part: CD271(red), CD49b(yellow)

Lower part: VEGFR2(red), VCAM(yellow)

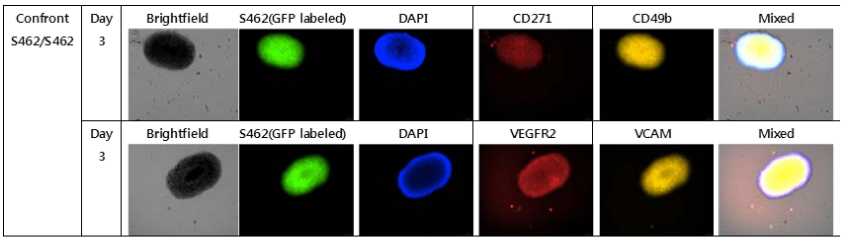

Figure 7. Immunostaining of S462-GFP – S462-GFP tumor confrontation for cell adhesion and endothelial markers.

Upper part: CD271(red), CD49b(yellow)

Lower part: VEGFR2(red), VCAM(yellow)

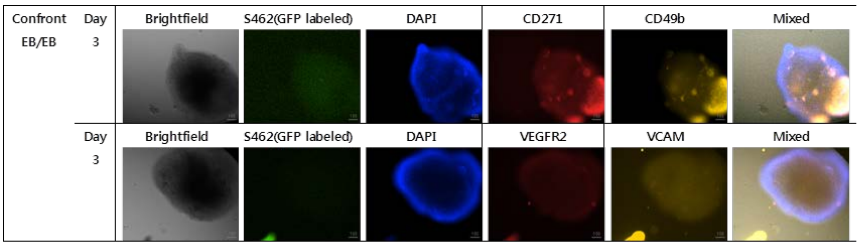

Figure 5. Immunostaining of EB-EB confrontations for cell adhesion and endothelial markers.

Upper part: CD271(red), CD49b(yellow)

Lower part: VEGFR2(red), VCAM(yellow)

Marker	CD271 (NGFR p75)		CD49b (Integrin)	
Cell type	**MSC**	**S462**	**MSC**	**S462**
MSC	78%	-	0%	-
S462	-	100%	-	100%
MSC/S462	80%	100%	0%	100%

Marker	VEGFR2 (endothelial cell marker)		VCAM (endothelial cell marker)	
Cell type	**MSC**	**S462**	**MSC**	**S462**
MSC	100%	-	62.5%	-
S462	-	22%	-	11%
MSC/S462	100%	44%	75%	22%

Marker	CD271		CD49b	
Cell type	**MSC+HUVEC**	**S462**	**MSC+HUVEC**	**S462**
MSC+HUVEC	44%	-	44%	-
S462	-	100%	-	100%
MSC+HUVEC/S462	80%	100%	40%	100%

Marker	VEGFR2		VCAM	
Cell type	**MSC+HUVEC**	**S462**	**MSC+HUVEC**	**S462**
MSC+HUVEC	100%	-	66%	-
S462	-	22%	-	11%
MSC+HUVEC/S462	100%	62.5%	62.5%	37.5%

Table 1. Quantification of spheres positive for endothelial markers.

Discussion

Using 3D models in tumor research has shown more in vivo like features than 2D cultures, and the use of human cells eliminates species differences. The development of increasingly complex 3D in vitro models which aim to recapitulate the tumor microenvironment, in terms of cell types and acellular constituents will further our understanding of the cell-cell and cell-matrix interactions occurring during tumor growth and invasion. 3D studies demonstrate different cell morphology and expression compared to 2D, resembling more closely cells in vivo and affirming a role for 3D models. Furthermore, they have the potential to provide a serious alternative to in vivo models, particularly in delineating molecular mechanisms underlying tumor growth and progression and drug action.

Our research shows that MSCs, when grown as spheres, affect tumor spheres towards the expression of endothelial markers. Whether this induction is due to a transition from a tumor stem cell into an endothelial cell type needs to be elucidated. However, such a transition

has been shown for glioblastoma and potentially contributes to tumor resistance against classical anti-angiogenic drugs.

The main benefit of our research is that we were able to show that tumor spheres in confrontation culture with embryoid bodies induce the expression of endothelial markers in the latter. While a similar effect has been shown for EBs from mouse embryonic stem cells, this is the first time that human EBs were used. Furthermore, I could show that the induction is already visible after 3 days of confrontation. However, whether this expression presents true neoangiogenesis remains to be elucidated. If so, it could be used as an fast assay for measuring angiogenesis and anti-angiogenic drug efficacies.

In order to use the confrontation assay for drug screening it is necessary to position the spheres at a standardized spot for high content screening. For this, we have tested a matrix-based printing array to selectively attach and array spheres and confrontation cultures. Our data show proof of principle of this approach.

The next step would be the testing of the endothelialization of EBs in confrontation cultures, which are fixed on an matrix-printed array.

Conclusion

1. Sphere technology can be used to analyze angiogenic induction by tumor cells.
2. Matrix printing facilitates arrangement of spheres and controlling of confrontation cultures for high content screening.
3. MSC may induce an endothelial transition in sarcoma cells.

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국 문 초 록

3 차원 구체를 이용한 신생 혈관 형성 분석법 연구

서울대학교 대학원

수의학과 전공

오 수 전

(지도교수 : Andreas Kurtz)

2차원 세포 배양 보다 3차원 세포 배양 시스템은 세포간에 일어나는 상호작용 및 세포와 간질 사이의 상호작용에 있어서 체내 배양에 유사한 점을 많이 가지고 있다. 그러므로 3차원 배양은 종양 혈관 생성 등에 관련된 약물 스크리닝이나 생체

적 작용의 분석 등의 체외 실험에 적합하다. 하지만 3차원 시스템의 단점은 아직까지 표준화된 고 컨텐츠 스크리닝이나 고속대량분석법이 없는 것이다.

체외 3차원 시스템 중에서 가장 정립되어 있는 시스템은 세포 구체를 부유시켜 배양하는 시스템이다. 이 연구에서는 세포 구체를 기본으로 하여 신생혈관생성 분석을 고 컨텐츠 스크리닝 기술로 실시할 수 있는 방법을 개발하였다. 고 컨텐츠 스크리닝은 세포의 고속대량분석이 가능하도록 하는 시각적인 방법이다.

신경섬유종 세포와 중간엽줄기세포, 그리고 유도분화줄기세포를 이용하여 각각 구체를 만들어 줄기세포의 구체에서 종양세포의 유도에 의해 내피세포 마커들이 유도되는지는 분석하는 것으로 신혈관생성의 모델을 구축하였다. 이 분석을 위하여 종양 구체와 줄기세포 구체들은 접촉한 상태로 같이 배양되었다. 그리고나서 이 모델이 고 컨텐츠 스크리닝에 적용가능한지를 테스트하여 보았고, EB에서 초기 내피세포형성 마커인 VEGFR2가 종양구체에 의해 유도되는 것을 확인하였다. 게다

가 고 컨텐츠 스크리닝에 반복해서 적용할 수 있도록 구체를 고정하고 선택할수 있는 방법으로 matrix printing 기법을 정립하였다.

결론적으로 이 연구에서는 구체를 바탕으로 한 3차원 혈관 생성유도를 정립하고, matrix printing을 기술화 하여 고 컨텐츠 스크리닝을 위한 confrontation culture를 조절하는 기술을 개발하였다.

주용어 : 3차원 배양, 신경섬유종, 고 컨텐츠 스크리닝

학번 : 2011-21699