Title: Fibroblasts cellular model for assessing efficacy of cancer treatments by SHH/PTCH pathway antagonists

Abstract: ABSTRACT Fibroblasts cellular model for assessing efficacy of cancer treatments by SHH/PTCH pathway antagonists. The present invention is in the domain of pharmacy and more specifically in skin cancer area and particular for Basal Cell Carcinoma (BCC). The present invention provides a cellular model targeting the Sonic Hedgehog/Patched (SHH/PTCH) pathway dysregulation or inappropriately activated as well as screening method using this cellular model to screen pharmacological compounds able to treat or prevent BCC lesions.
Fibroblasts cellular model for assessing efficacy of cancer treatments by SHH/PTCH pathway antagonists

The present invention is in the domain of pharmacy and more specifically in skin cancer area and particular for Basal Cell Carcinoma (BCC). The present invention provides a cellular model targeting the Sonic Hedgehog/Patched (SHH/PTCH) pathway dysregulation or inappropriately activated as well as screening method using this cellular model to screen pharmacological compounds able to treat or prevent BCC lesions.

The Hedgehog pathway is normally active during embryonic development and plays a central role in cell differentiation and proliferation. Inappropriate activation or dysregulation of the Hedgehog pathway is believed to play a critical role in the proliferation and survival of certain cancer cells, including in basal cell carcinoma and medulloblastoma.

Known pathway-activating mutations include those that impair the ability of PTCH, a transporter-like Hh receptor, to restrain Smo (SMO) activation of transcriptional targets via the GLI family of latent transcription factors. Binding of Hh ligand to PTCH is functionally equivalent to genetic loss of PTCH, in that pathway activation by either requires activity of SMO, a seven transmembrane protein that binds to and is inactivated by the pathway antagonist, cyclopamine.

The implication of PATCHED pathway activation in several cancer conditions, most notably in BCCs, has motivated much effort to set up experimental systems to assess the inhibitory activity of small molecules.

The existing systems to measure activation or inhibition of the activated SHH/PTCH pathway, are based on cell lines from human or mouse origin. Theses cells can schematically be classified in two categories destined to measure i-) endogenous cellular events after treatment; these events include triggering of a differentiation process and modulation of gene expression, notably of those genes known as transcriptional targets of pathway activation; ii-) cell lines engineered to report pathway activation / inhibition after transient or permanent introduction of reporter constructs made of responsive DNA driving a reporter gene. Cell lines developed so far are:

- Human normal primary keratinocytes and fibroblasts in reconstructed skin where expression of GLI1 and GLI2 mRNA have been measured to demonstrate inhibition by the small Robotnikinin molecule of SHH/PTCH pathway activation by SHH (Stanton et al, 2009)
Healthy human primary keratinocytes from patients with nevoid basal cell carcinoma or Gorlin syndrome have been isolated to mimic the somatic loss of one \textit{PATCHED} allele in sporadic BCC epidermal cells (Brellier et al., 2008a).

However, the above described cell lines have some disadvantages. Most of cell lines are not stable in the sense that after several passages the expression of inserted genes decreases strongly or is shut down. Either those cell lines are not sufficiently robust to be efficient and sensitive to be used in a drug screening as a model. None of the cited prior art provides a system to allow a simple detection of activation in human skin cells, including keratinocytes and fibroblasts.

Thus, there is a need for developing a human cell line easy to produce and to use, efficient, being a relevant model and sensitive for the screening or assessment of molecules libraries.

The inventors have developed a new cell line providing strong advantages. Indeed, the present invention provides an immortalized cell line of human skin fibroblasts which responds to pathway stimulation without the need to introduce an exogenous reporter cassette using genetic engineering, thus that cell line is characterized in that reporter gene is endogenous with its own elements of response to pathway activation. The reporter capacity of this human cell line is stable overtime, particularly even after a high rate from passages in tissue culture.

In preferred embodiment of invention, the immortalized reporter fibroblast cell line according to the invention, express the \textit{PTCH} protein and/or other members of the pathway that are necessary to convey responses to agonists and antagonists of the said pathway as shown in figure 3. Said cell line is immortalized by retroviral transduction of pLE6/E7SN. In addition, this cell line is produced in standard cell culture medium without addition of sophisticated goodies but only ready to use elements.

The invention provides also a process for obtaining immortalized human fibroblasts as describe above, comprising the following steps:

- isolated human primary Fibroblasts from healthy skin from individual having NBCCS or Gorlin syndrome
- immortalize the cell line by retroviral transduction with pLE6/E7SN
- select immortalized cell line with a medium of selection
- check for attenuation of P53 expression
- check for growth properties,

The invention provides also a drug screening method, wherein said immortalized fibroblast cell line as described above is used to screen. In a preferred embodiment of the invention, the drug screening method comprises the following steps:

a). bringing one samples of immortalized fibroblasts cell line as described above into contact with one or more of the test compounds;
b). measuring the expression of endogenous reporter gene expression, namely by quantitative measurement of accumulation of the GLI1 mRNA, preferentially by Q-RT-PCR
c). selecting the compounds for which a modulation of the expression of the activity of endogenous reporter gene is measured in b) and compared with no drug mixture.

In a preferred embodiment, the drug identified and/or selected according to the drug screening method as described above is an anti-tumor drug.

The present invention also provides an in vitro method for the screening of candidate compounds for the preventive and/or curative treatment of cutaneous cancer and preferentially basal cell carcinoma.

The invention regards also to a drug obtainable with the drug screening method as described above.

Detailed description

Basal Cell Carcinomas (BCC) of the skin is the commonest human cancer. BCCs derive from epidermal keratinocytes. The great majority of BCCs occurs on photo-exposed skin due to ultraviolet induced mutagenesis. The steadily rising incidence of BCCs in the last decades is attributed to increasing enthusiasm for recreational sun exposure. Although BCC rarely metastasize, they can result in local destruction and invasion of underlying tissues and consequently, life threatening complications. BCC are usually treated by local surgical excision, topical chemotherapy, photodynamic therapy, but, according to tumor size, location and frequency, there may be considerable aesthetic sequelae. Thus, drawbacks of current BCC treatments strongly support the need for pharmacological innovations that should specifically target the SONIC in so far as inappropriate and constitutive activation of this
pathway is associated with the vast majority of BCC (see below). In several cancers including BCCs, it has been suggested that accumulation of mRNA corresponding to target genes of the pathway depends on interactions of tumor cells with fibroblasts of the microenvironment. Furthermore, molecules that target the SHH pathway could also be of interest in the treatment of other / non-BCC cancer conditions where the influence of stromal microenvironment (e.g. melanoma, pancreas, oesophagus, liver, prostate, lung, muscle, colon) is thought to play a role in inappropriate activation of the SHH/PTCH pathway (for review see, (Scales and de Sauvage, 2009)).

The SHH/ PATCHED pathway

The SHH/PTCH signaling pathway is essential during embryogenesis and development where it controls cell fate by modulating proliferation and differentiation. Animal models, notably the fruit fly drosophila melanogaster, have shown that at specific stages of development, some cells produce and emit a signal, the Hedgehog molecule (HH), which, in turn, is received by target cells. In vertebrates, the family of Hedgehog molecules is composed of Sonic Hedgehog, SHH, Desert Hedgehog, DHH, and Indian Hedgehog, IHH. Target cells (of these ligands) express PATCHED (PTCH), a putative twelve pass transmembrane protein acting as the receptor of HH molecules. When HH molecules are not expressed and / or not secreted at the vicinity of target cells, PTCH acts as a repressor of the pathway by inhibiting another transmembrane protein called SMOTHENED (SMO). SMO is a putative seven pass transmembrane protein apperrented to G-protein coupled receptors. The inhibition of SMO by PTCH is relieved in the presence of HH molecules bound to PTCH. De-repression of SMO leads to activation of transcription factors of the GLI family (named GLI 1, 2 and 3) that activate (GLI1 and 2) or repress (GLI3), the transcription of their target genes. Interestingly, PTCH1 is a transcriptional target of GLI1 and GLI2 factors (Scales and de Sauvage, 2009).

The importance of the SHH / PTCH pathway is illustrated by severe diseases due to mutations affecting its integrity at different levels. Notably, in the human, heterozygous mutations in the PTCH1 gene are responsible for the dominantly inherited genetic syndrome called nevoid basal cell carcinoma syndrome (NBCCS or Gorlin syndrome). NBCCS patients are highly prone to BCCs that generally (about 50 % cases) present with a loss of heterozygosity in the PTCH1 locus. In Gorlin patients, more than 50% BCCs also bear mutation in the tumor suppressor gene TP53, suggesting some cooperation of the P53 and the SHH/PTCH pathways toward development of BCCs. Very interestingly, the two PTCH1 alleles are also lost in most sporadic (general population) BCCs; in the latter case, again, the
two TP53 alleles are found mutated in 10 to 50% sporadic BCCs. 20-30% sporadic BCCs are mutated in both TP53 and PTCH1. In both NBCCS and sporadic BCCs, inactivation of PTCH results in constitutive activation of the pathway with accumulation GLI1 and GLI2 mRNAs (Dahmane et al., 1997; Unden et al., 1996). In contrast to sporadic BCCs that almost exclusively develop in photo exposed skin area, about 40% NBCCS BCCs develop in non exposed skin. In addition, our previous observations have indicated that primary NBCCS primary fibroblasts isolated from healthy skin expressed a transcriptome resembling that characterized in fibroblasts associated to sporadic carcinomas (CAF) (Valin et al., 2009; Valin and Magnaldo, 2008). Together, these observations strongly support the idea of a strong contribution of dermal fibroblasts in carcinoma development in NBCCS patients.

The implication of the SHH/PTCH pathway activation in several cancer conditions, most notably in BCCs, has motivated much effort to set up experimental systems to assess the inhibitory activity of small molecules.

The existing systems of activity measure are based on cell lines from human or mouse origin. Theses cells can schematically be classified in two categories destined to measure i) endogenous cellular events after treatment; these events include triggering of a differentiation process and modulation of gene expression, notably of those genes known as transcriptional targets of pathway activation; ii) cell lines engineered to report pathway activation / inhibition after transient or permanent introduction of reporter constructs made of responsive DNA driving a reporter gene. Cell lines developed so far in the prior art reveals that none of those system allows simple detection of activation in human epidermal keratinocytes.

To provide a simple detection system, the inventors have worked to develop a human cell line in the respect of the following specifications (i.e. what we need for easy, efficient, relevant, sensitive assessment of molecules libraries):
   - human cells,
   - skin cells,
   - growth in standard medium,
   - needing no feeders,
   - genetic stability and activity of the reporter gene a long period of time, including long term expression over cell generations;
   - highly sensitive to activation; the cell line must report activation at doses closed to ligand (SHH) affinity, thus at the nM order.
To fulfil these specifications, the strategy was to use a human cell strain derived from healthy individuals having NBCCS or Gorlin syndrome. The choice of NBCCS cell was dictated by the fact that our previous investigations have suggested that NBCCS (heterozygotes for the PATCHED, suggesting the partial loss of the PTCH repressor activity) cells are particularly sensitive to pathway activation. Thus, we hypothesized that cells from those NBCCS patients could certainly constitute a valuable tool to measure activation and inhibition of the activation with a high sensitivity. As indicated in prior art, no such line is available, neither from academic nor from commercial sources. The rationale of using natural endogenous responses to SHH-like agonists stems from i) the physiological relevance, i.e. good sensitivity of the cell line relying on natural elements of transcriptional control (control regions of SHH-regulated genes), ii) avoiding the use of direct tandem repeats of GliBS (n=8) upstream the Firely luciferase gene as described (Sasaki et al., 1997). Direct tandem repeats of GliBS are known to be very unstable; as in many other laboratories, all attempts to construct a reporter cell line using these sequences have failed. Concerning the easiness of growth in standard non sophisticated culture media, we decided to abrogate or at least to attenuate, the expression of the tumor suppressor gene TP53.

In the specific case of NBCCS cells, other advantages of abrogating P53 stem from our and others reports indicating that: i) P53 stabilisation after a single UVB irradiation is higher and prolonged in NBCCS compared to control cells (Brellier et al., 2008b); ii) there is a mutual inhibition of gli1 and P53 (Stecca and Ruiz i Altaba, 2009); iii) 20-30 % BCCs bear mutations in both TP53 and PATCHED suggesting that attenuation of P53 could enhance sensitivity to PTCH pathway activators.

Thus, it is reasoned that abrogation or attenuation of the P53 pathway using E6-E7 oncogenic proteins of Human Papilloma Virus 16 (HPV16) would favor activation of the SHH/PTCH pathway in human skin cells, including fibroblasts. Preferably, it was decided to use human primary fibroblasts from healthy skin (non photo exposed non tumoral) from individuals having NBCCS or Gorlin syndrome, after transformation with the E6-E7 oncogenic proteins (NBCCS 6 E6/E7 fibroblasts).

Thus, the present invention provides an immortalized cell line of human fibroblasts from healthy individuals having NBCCS or Gorlin syndrome expressing an enhanced and stable response toward PTCH pathway activation overtime, particularly even after a high rate from passages in tissue culture.
It meant by stable expression of the endogenous reporter gene overtime that after high number of passages the level expression is the same as the initial level without recombination or lost of chromosomal material, as this can be observed in cell lines that express an exogenous reporter gene (i.e. Luciferase) under the control of GLI binding sequence in direct repeat (X8) tandem configuration (Sasaki et al, 1997).

In a preferred embodiment of the invention, the immortalized fibroblasts from healthy individuals having NBCCS or Gorlin syndrome cell line according to the invention express the PTCH protein and/or other members of the pathway that are necessary to convey responses to agonists and antagonists of the said pathway as shown in figure 4. Said cell line is immortalized by retroviral transduction. The skilled in the art is familiar with retroviral transduction techniques and all of them are applicable to the present invention. Any kind of retrovirus can be used such as Moloney murine leukemia virus (MoMLV), lentivirus, Eptein-Barr virus (EBV).... MoMLV is preferred for high performance of infection in human primary fibroblasts (Quilllet et al, 1996). The retroviral transduction of pLE6/E7SN is preferred in this context.

In addition, this cell line is produced in standard culture medium (DMEM based containing non sophisticated, ready to use goodies). In addition the cell line stands temporary serum starvation (0.5 % serum) which allows avoiding interference with the activity response. Thus, response of cells to pathway activation in a quasi defined medium providing the advantage of growing cells in medium which does not interfere with activity response. The invention provides thus a robust model with expected or calibrated response which avoids any interfering factors.

The invention provides also a drug screening method, wherein said immortalized fibroblast cell line as described above is used to screen. The invention relates to an in vitro screening method of the PTCH pathway inhibitors for treating skin cancer and preferably BCC, comprising determining the capacity of said drug to inhibit or down regulate expression or biological activity of the PTCH pathway.

In a preferred embodiment of the invention, the drug screening method comprises the following steps:

a), bringing one samples of immortalized cell line as described above into contact with one or more of the test compounds;
b). measuring the expression or the activity of the reporter gene mRNA, preferably GLI1

c). selecting the compounds for which a modulation or of the expression of the reporter gene, preferably GLI1 thereof, is measured in b) and compared with no drug mixture.

In the context of the invention, any other gene responsive to the pathway activation (PATCHED1, CCD1, BCL2, SNAIL etc.) known by the skilled artisan is applicable. In a preferred embodiment, the reporter gene is GLI1.

The present invention provides tools for selecting SHH/PTCH pathway modulators. Those modulators are activators or inhibitors.

In a preferred embodiment, the drug identified and/or selected according to the drug screening method as described above is an anti-tumor drug. The reporter gene, preferably GLI1, is first activated and inhibition efficacy of one or several drug candidates (isolated or in a mixture) is assessed, preferably with increasing concentration. The examples provide an illustration with a particular embodiment in GLI1 mRNA accumulation as reporter model.

FIGURES

The following figures illustrate the invention:

Figure 1: Schematic map of the LE6E7 SN proviral construct. LTR5', long terminal repeat 5'. E6E7, sequence of the human papilloma virus 16 encoding the E6 and E7 transforming proteins. Neo, neomycin phosphotransferase gene conferring resistance to G418 antibiotic. LTR 3', long terminal repeat 3'.

Figure 2: Transformation of the control or NBCCS6 fibroblasts cell line by the E6/E7 oncoproteins

Figure 3: RT-Q-PCR analysis of expression of mRNAs encoding actor proteins of the SHH/PTCH_pathway_shows that NBCCS 6 E6/E7 fibroblasts express essential actors of the PTCH/SHH pathway

Figure 4: Comparative Q-PCR analyses of GLI1 mRNA accumulation after treatment of fibroblasts cell lines with purmorphamin. A, CTRL E6/E7 cells, B, representation of the response of CTRL cell (shown in A) to the agonist in comparision to the response of NBCS
E6/E7 cells. Note that NBCCS 6 E6/E7 exhibit a 7 x times higher response than CTRL cells in the same experimental conditions.

EXAMPLES

The examples which follow illustrate the invention without limiting the scope thereof.

Example 1: Materials and Methods

Cell Culture

Human primary fibroblasts (named CTRL or NBCCS) were isolated from a healthy non photo-exposed skin biopsy of either a control patient or a patient with characteristic NBCCS after informed consent (Otto et al., 1999; Valin et al., 2009)

Cell transformation and selection

The CTRL and NBCCS6 cell lines were then immortalized by retroviral transduction with pLE6/E7SN resulting in CTRL and NBCCS-E6-E7 (figure 1) (Halbert et al., 1991, 1992). Cells were grown at 37 °C in a humified atmosphere containing 5 % CO2, DMEM medium, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.125 µg/ml amphotericin B, 2mM L-Glutamine, 1 mM Sodium pyruvate, 1x non essential amino acids.

G418 is an aminoglycoside antibiotic similar in structure to gentamicin B1, produced by Micromonospora rhodorangea. G418 blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells. Resistance to G418 is conferred by the Neomycin resistance gene (neo) from Tn5 encoding an aminoglycoside 3'-phosphotransferase, APH 3' II.

Selection in mammalian cells is usually achieved in three to seven days with concentrations ranging from 200 to 1000 µg/ml (Arnaudeau-Begard et al., 2003).

Real time quantitative PCR

Total RNA was extracted with RNeasy® Mini kit according to manufacturer’s instructions (Qiagen, Hilden, Germany). Reverse transcription was performed on 1 µg total RNA with Superscript II Reverse Transcriptase (Roche Applied Science, Basel, Switzerland) by using random primers. Q-PCR was carried out using the 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).
Example 2: Transformation of the control or NBCCS6 fibroblasts cell line by the E6/E7 oncoproteins

Effective transformation of the CTRL and NBCCS6 cell line was assessed by analysing their growth properties and attenuation of expression of the P53 tumor suppressor protein. Figure 2 shows a western blot analysis of P53 in cell extracts prepared from preconfluent (about 80%) primary fibroblasts (CTRL, or NBCCS6), before or after transformation using the E6E7 encoding retroviral vector (CTRL E6/E7, NBCCS6 E6/E7). The western blot (figure 2) shows the drastic decrease in the amount of the P53 protein. GAPDH is a control of loading.

Example 3: RT-Q-PCR analysis of expression of mRNAs encoding actor proteins of the SHH/PTCH pathway shows that NBCCS 6 E6/E7 fibroblasts express essential actors of the PTCH/SHH pathway

As shown in figure 3, NBCCS 6 E6/E7 fibroblasts express essential actors of the PTCH/SHH pathway. RT-Q-PCR was performed from total cDNAs prepared from the indicated cells. CTRL, human primary dermal fibroblasts before or after CTRL E6/E7 immortalization by pLE6E7 retroviral transduction. NBCCS6, primary fibroblast from Nevoid Basal Cell Carcinoma Syndrome patient # 6 before or after (NBCCS6 E6/7) immortalization by pLE6E7 retroviral transduction.

Example 4: Comparative Q-PCR analyses of GLI1 mRNA accumulation after treatment of fibroblasts cell lines with purmorphamin.

To evaluate small Molecules Modulators of the SHH/PTCH pathway, the Purmorphamine (SMO agonist) was used. Purmorphamine (SMO agonist) was diluted in DMSO at stock concentrations of 50 mM and 10 mM, respectively. To avoid side effects and toxicity, the final concentration of DMSO was fixed to 0.1 % DMSO.

As shown in figure 4, the comparative Q-PCR analyses of GLI1 mRNA accumulation after treatment of fibroblasts cell lines with purmorphamin are represented in figure 4 A, CTRL E6/E7 cells, Figure 4B, is the representation of the response of CTRL cell (shown in A) to the agonist in comparison to the response of NBCS_E6/E7 cells.
It should be noted that NBCCS 6 E6/E7 exhibit a 7 x times higher response than CTRL cells in the same experimental conditions.

References


CLAIMS

1. Immortalized cell line of human fibroblasts from healthy individual having NBCCS or Gorlin syndrome with a natural endogenous GLI1 gene with stable expression and inducibility overtime.

2. Cell line of immortalized fibroblasts according to claim 1, wherein expression is stable even after a high rate from passages in tissue culture.

3. Cell line of immortalized fibroblasts according to claim 1, characterized in that reporter gene is endogenous with its own elements of response to pathway activation.

4. Cell line of immortalized fibroblasts according to claim 3, characterized in that it expresses PTCH protein.

5. Cell line of immortalized fibroblasts according to claim 3, characterized in that it expresses other members of the pathway required for inhibition and activation of the said pathway.

6. Cell line of immortalized fibroblasts according to claim 1 characterized in that it is immortalized by retroviral transduction of pLE6/E7SN.

7. Cell line of immortalized fibroblasts according to claim 1, characterized in that it is produced without using feeder cells.

8. Process for obtaining immortalized human fibroblasts as describe in claim 1 to 7, comprising the following steps:

   - isolated human primary fibroblasts from healthy individual having NBCCS or Gorlin syndrome
   - immortalized cell line by retroviral transduction with pLE6/E7SN
   - select cell line expression with a medium of selection
   - check for attenuation of P53 expression
   - check for growth properties,

9. Drug screening method, characterized in that it uses the immortalized fibroblasts according to claim 1 to 7.

10. Drug screening method according to claim 9, comprising the following steps:
a. bringing one samples of immortalized fibroblasts cell line as described in claims 1 to 7 into contact with one or more of the test compounds;
b. measuring the expression or the activity of reporter gene mRNA, preferably GLI 1
c. selecting the compounds for which a modulation of the expression of reporter gene, preferably GLI1, is measured in b) and compared with no drug mixture.

11. Drug screening method according to claim 10, wherein the drug identified is an anti-tumor drug

12. In vitro method for screening for candidate compounds for the preventive and/or curative treatment of cutaneous cancer, comprising the following steps:
   a. bringing one samples of immortalized fibroblasts cell line as described in claims 1 to 7 into contact with one or more of the test compounds or a mixture of compounds;
b. measuring the expression or the activity of endogenous reporter gene expression
c. selecting the compounds for which a modulation of the expression or of the activity of reporter gene, is measured in b) and compared with no drug mixture.

13. Drug obtainable with the drug screening method according to claims 9 to 13.
Figure 3:

- **PTCH1**
- **SMO**
- **GLI1**
- **GLI2**
- **GLI3**
Figure 4
**INTERNATIONAL SEARCH REPORT**

**International application No**

PCT/EP2012/068777

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**A. CLASSIFICATION**

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**ADD.**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

C12N  C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, Sequence Search

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**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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<td>US 6 458 593 BI (MUSICIK JAMES R [US] ET AL) 1 October 2002 (2002-10-01) claims 20-21 the whole document</td>
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<td>WO 2010015618 A1</td>
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