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Tet responsive and adenovirus based constructs for regulated *in vivo* expression of *Lefty*

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1. ABSTRACT

Regulated expression of *Lefty/Ebaf* during embryogenesis is required for development of body asymmetry. A tight regulation of *Lefty* also contributes to the menstrual tissue shedding in humans. In order to replicate this regulated expression, we have developed a tet-on system and an adenovirus driven model. To drive the expression of *Lefty*, we have placed *Lefty* under control of a tetracycline-responsive promoter which responds to a sequence variant of the reversed tet transcriptional activator (rtTA), rtTA2^{S}-M2. In this model, *Lefty* is regulated by the dose of doxycycline. In the adenovirus driven system, *Lefty* is regulated by the number of adenoviral particles These model systems would be suitable for understanding the dose-dependent biologic role of *Lefty in vivo*.

2. INTRODUCTION

Current *in vivo* models of gene transfer, still have important limitations. Many occur because the promoters that are used often initiate gene expression *in vivo* and subsequently, drive gene expression in a largely constitutive fashion. Superimposing growth and/or developmentally-related abnormalities on the phenotypic changes that would be caused by the gene product in an adult animal makes the interpretation of data difficult. These models also generate phenotypes that are accurate representations of the effects of chronically expressed proteins, but *in vivo*, genes are used in an intermittent or dose dependent fashion. Thus, many overexpression strategies have a limited ability to model waxing and waning which occur during organ growth and development and during disease processes. Ideal models of *in vivo* gene

Table 1. Primers used in PCR

Name	Sequence
Lefty specific hmL811-5'	GACCTGCAGGGGATGAAGTG
Lefty specific hmL1010-3'	CTGGTCCTGCCTCCCTCCTT
18SrRNA-5'	AGGCCATGATTAAGAGGGAC
18SrRNA-3'	CGCTCCACCAACTAAGAAC
pK18-IRES specific K18IRES(F)	GCTTTACATGTGTTTAGTCG
Activator specific rtTA2S-3'	GCTCCTGTTCCTCCAATACG

expression drive conditional, organ-specific and dose dependent overexpression of genes in the target tissue. Three approaches are needed to achieve this goal. The model should be regulated by an external agent that, when administered, will turn on the gene of interest, preferably in a dose dependent fashion. The "on" systems are preferred over the "off" system because of rapid kinetics of induction. Second, over-expression of the transgene should be confined to the organ and preferably the specific cell types that normally express the gene and it should be susceptible to induction during development and adulthood. Third, the system should not allow low-level basal expression of the transgene when the inducer is absent. Therefore, there is a need to suppress this background level of expression. In an attempt to address these limitations, systems have been developed in which gene expression can be externally regulated. Early attempts focused on a variety of approaches including the use of steroid-inducible and metallothionein-based promoter systems (1). More recent approaches have used tet-regulatory systems based on wild-type and mutated tetracycline transactivator fusion proteins (2). Subsequent studies have demonstrated that tet-based systems can be externally regulated after using standard transgenic technology (2-3). Moreover, the leakage in the Tet-on system has been addressed by developing a mutated promoter, rtTA2^S-M2 (4) and suppression of the background by use of a Tet silencer system, a fusion protein containing the tet repressor and the KRAB-AB domain of the kid-1 transcriptional repressor, which is inhibited by doxycycline.

Lefty regulates embryogensis and participates in menstrual process in a dose dependent manner. To examine the dose dependent effect of *Lefty in vivo*, in this report, we have developed two systems. One is based on driving the *Lefty* gene expression, in a dose dependent manner, by the dose of the doxycycline. In the other model, *Lefty* is regulated by the number of adenoviral particles.

3. METHODS

3.1. Materials

All chemicals were from Sigma-Aldrich company (St Louis, MO) or Fisher Scientific (Pittsburgh, PA). The BetaBlue Staining Kit was purchased from EMD Biosciences, Inc. (San Diego, CA). The goat anti-*Lefty* polyclonal antibody, donkey anti-goat IgG-HRP and goat anti-rabbit IgG-HRP secondary antibodies were from Santa Cruz Biotech Inc. (Santa Cruz, CA). The rabbit polyclonal antibody to β-actin was from Abcam, Inc. (Cambrige, MA). Goat ABC Staining System was from Santa Cruz Biotech Inc. (Santa Cruz, CA). Pre-stained protein ladder was from Life Technologies, Inc. (Rockville, MD).

3.2. Isolation and culture of decidual cells

Decidual cells were obtained from decidual membranes of a term placenta after cesarean section. Decidual tissues were collected by mechanical stripping from placental membranes and were digested twice by collagenase P (250 μg/ml) for approximately 30 min at 37^{o}C. Then, individual cells were obtained by trypsinizing the tissue at 37^{o}C for 5 min. Cells were cultured in 100 mm dishes in presence of RPMI 1640 medium supplemented with 10% fetal bovine serum and passaged two times before use. Approval of institutional Review Board of Stony Brook University was obtained for the isolation and use of decidual cells.

3.3. Preparation of DNA constructs

Plasmid DNA and PCR products digested with restriction enzymes were separated by electrophoresis in agarose gels. DNA fragments were cut out and isolated using Geneclean II Kit (BIO 101, CA) All constructs were checked for correct orientation of the inserts by restriction enzyme digestion. The sequence of DNA fragments derived from PCR was confirmed by sequencing.

3.4. Cell culture and transfection

293 cells were maintained in DMEM medium (Cellgro) supplemented with 5% FBS (Life Technologies, Inc., Rockville, MD) and 1% antibiotic-anti-mycotic (Life Technologies, Inc., Rockville, MD). Plasmid DNAs were introduced into adherent 293 cells in 60 mm plates by the standard calcium phosphate-mediated transfection. Transfected cells were washed once in serum-free DMEM and left in fresh prewarmed media without serum, with or without doxycycline for 44 hr. Stromal fibroblasts from human placenta were cultured in RPMI medium with 4% FBS, in presence of hormones (0.1 μM medroxyprogesterone acetate, 36 ng/ml 17 beta estradiol). Fibroblasts were either transfected with DNA constructs, transduced with *Lefty* adenovirus (Ad-*Lefty*) or control empty adenovirus (Ad-CMV), or left in experimental media as control.

3.5. Quantitative PCR

We used quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to quantify *Lefty* and rtTA2^S-M2 mRNAs. Real time PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) and DNA Engine Opticon system (MJ Reserach, Waltham, MA). Quantitative PCR (qPCR) was performed in a total volume of 20 μl using primer sets shown in table 1 and aliquots (3 μl) of reverse transcription products (cDNAs), which were synthesized using 0.4-0.8 μg of DNase-treated total RNA, random primers, and ProtoScript First Strand cDNA Synthesis Kit (New England BioLabs, Inc.) in 10 μl volume (after reverse transcriptase inactivation, cDNAs were diluted to 25 μl with water and those were used in qPCR). For normalization of data, control PCRs were performed using 18S rRNA-specific primers and with diluted templates.

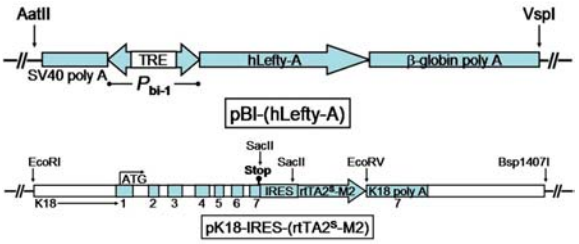

Figure 1. Constructs for tet-regulated expression of *Lefty*. The inducible system requires two genetic constructs: pBI-(h*Lefty*-A) and pK18-IRES-(rtTA2^S-M2). Upper panel: The main elements of pBI-(h*Lefty*-A), the *Lefty* vector, are the human *Lefty*-A-coding region, β globin polyadenylation sequence, a bidirectional minimal CMV plus tet-responsive element (TRE) synthetic promoter P_{bi-1}. Lower panel: Activation of P_{bi-1} promoter requires a tet-controlled activator such as rtTA2^S-M2, which is expressed from pK18-IRES-(rtTA2^S-M2) using the K18 gene transcriptional elements and the encephalomyocarditis virus (ECMV) internal ribosome entry site (IRES).

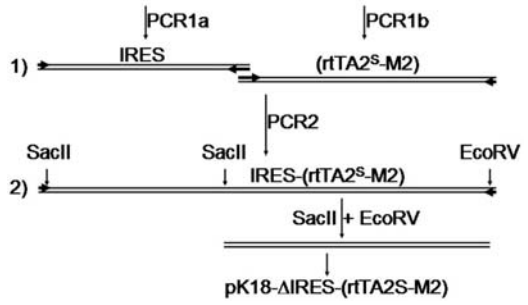

Figure 2. Strategy for cloning of the activator, rtTA^S-M2, in a K18 vector by a two step PCR approach. This approach allowed cloning of the rtTA2^S-M2, 5 basepairs downstream of the last ATG codon of IRES. The final step of creating pK18-IRES-(rtTA2^S-M2) from ΔIRES-(rtTA2^S-M2) by adding 550 bp SacII-SacII fragment (IRES) from pK18-IRES is not shown. K18: human keratin 18 gene, IRES: encephalomyocarditis virus (ECMV) internal ribosome entry site, rtTA2^s-M2: a sequence variant of rtTA, a reversed Tet transcriptional activator.

3.6. SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

The cell lysates (12-15 μg protein/lane) were fractionated in a 12% denaturing gel together with pre-stained protein ladder. Proteins were subsequently blotted onto nitrocellulose membrane in a Mini-Trans-Blot apparatus (Bio-Rad Laboratories, Hercules, CA). The blots were stained with the polyclonal antibodies to *Lefty* and actin specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). at a concentration of 2 μg/ml. The secondary antibody used was anti-IgG-HRP (Santa Cruz Biotechnology, CA). Bands were detected by chemiluminescence as described by the manufacturer.

3.7. β-galactosidase staining and immunostaining of *Lefty*

Cells were fixed for 30 min at 4^{o}C with 2% paraformaldehyde in phosphate-buffered saline (PBS) containing 2 mM $MgCl_2$, 5 mM EGTA (pH 8.0), and 0.02% NP-40. Samples were then washed and stained for 16 h at 37^{o}C for β-galactosidase in a staining buffer containing 1 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% SDS, and 0.02% NP-40. Cells were fixed in 10% buffered formalin for five minutes and stained with *Lefty* specific antibody. Cells were washed and incubated with peroxidase specific secondary antibody, followed by development in a mixture of DAB-H_2O_2.

3.8. Statistical analysis

Values were expressed as means±SEM. Group means were compared by Students *t* test procedure.

RESULTS AND DISCUSSION

4.1. Generation of *Lefty*-A Tet-On constructs

Standard overexpression approaches are limited by their inability to regulate gene expression in a dose-dependent manner, resulting in development-dependent and -independent phenotypic manifestations. We have used the keratin 18 gene (K18) fused to an internal ribosomal entry sequence (IRES) of encephalomyocarditis virus and a sequence variant of the reverse tetracycline transactivator (rtTA2^S-M2) to create a regulated *Lefty* expression system. In this model, *Lefty* expression is induced, in a dose dependent manner, with doxycycline (Dox). The original rtTA system, has three drawbacks. First, rtTA is less sensitive than tTA (tet-off) system to induction by Dox. Second, a background level of transgene expression is seen in the absence of Dox and third, high expression of the transactivator is toxic to mammalian cells. These problems can be circumvented through the use of rtTA2^S-M2, a sequence variant of rtTA that is more stable in eukaryotic cells, causes no background expression in the absence of Dox and is not toxic. Moreover, the rtTA2^S-M2 functions at a 10-fold lower Dox concentration than rtTA.

The constructs that were developed are illustrated in Figure 1. We have chosen to express the transactivator from the K18 gene promoter for several reasons. At this time, there is a paucity of epithelium-specific promoters and those that might be good candidates have not been well characterized. The K18 gene is expressed in epithelia and a K18-based vector, pK18-IRES, tends to provide position-independent, copy number-dependent, and tissue-specific expression (5). Thus, this vector would allow determining the effects of *Lefty* overexpression in all epithelial lined organs including the uterus, where *Lefty* is distinctly regulated during menstruation (6).

The *Lefty* conditional system is based on the transfection of two constructs (Figure 1). A Lefty vector, pBI-(hLefty-A), allows expression of Lefty under the control of a tet activator (rtTA2^S-M2) produced from pK18-IRES-(rtTA2^S-M2). For efficient expression of the rtTA2^S-M2 protein from bicistronic mRNA, its ORF was placed 5 basepairs (personal communication, Robert Oshima) downstream of the last ATG codon of IRES in two-step PCR (Figure 2). IRES (600bp) and rtTA2^S-M2 (760bp) was amplified with specially designed primers (IRES PCR1a - with primers IRES5': aggcattaagccagcagaag

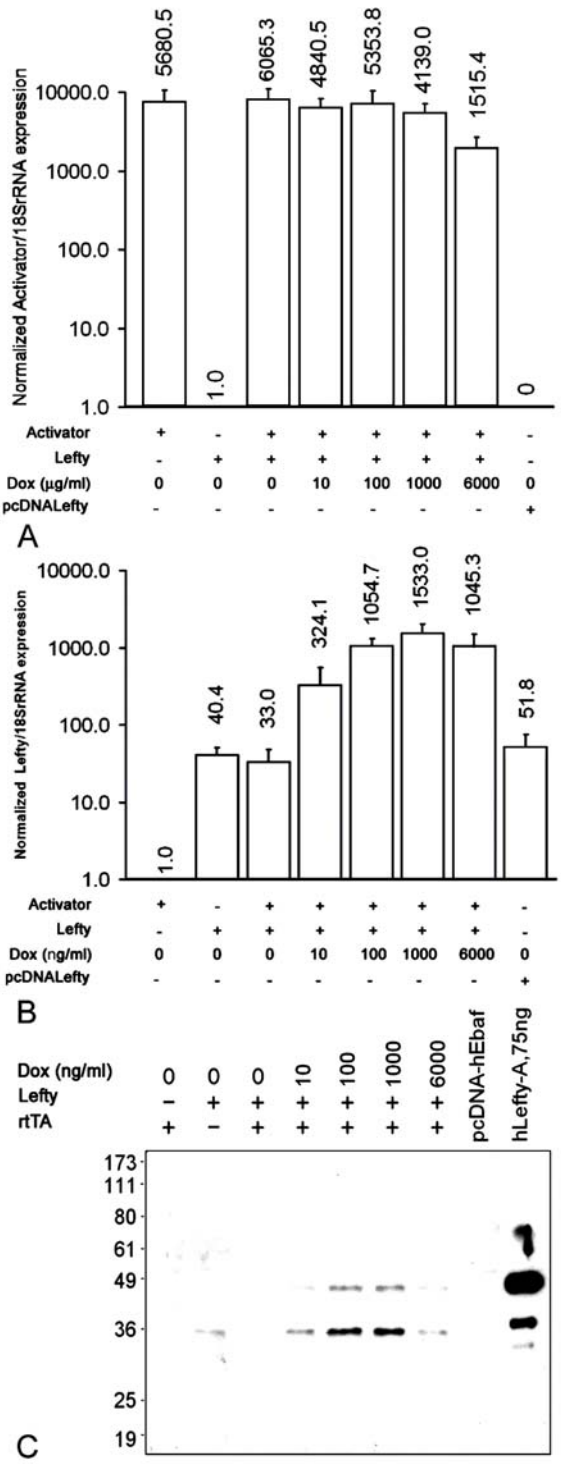

Figure 3. Testing the *Lefty* and rtTA (rtTA2^S-M2) constructs *in vitro*. Constructs with *Lefty* and rtTA (activator) were introduced into 293 cells, which were incubated in serum-free media with various doses of doxycycline (Dox). 44 hr post-transfection, cells and their media were harvested for total RNA isolation.A: qRT-PCR for activator, rtTA2^S-M2 B: qRT-PCR for *Lefty*. C: Immunoblot analysis of concentrated culture media of transfected cells for Lefty. Values are expressed as means±SD.

and IRES3: tctagacatattatcatcgtgtttttcaaagg; rtTA2^S-M2 PCR1b-with primers rtTA5:cacgatgataatatgtctagactggacaagagc and rtTA-RV-3': tgtcgatatccttacttagttaccc) to produce 21 basepair-overlapping DNA fragments. The two fragment were used as template in a second PCR (Figure 2-PCR2) with the left primer for IRES (IRES5') and the right primer for rtTA2S-M2 (rtTA-RV-3') to produce a single IRES-(rtTA2^S-M2) fragment. The fragment was digested with SacII and EcoRV, and 800 bp SacII-EcoRV fragment (ΔIRES-(rtTA2^S-M2)) was cloned at corresponding sites in pK18-IRES. This process replaced IRES in pK18-IRES with ΔIRES-(rtTA2^S-M2). The sequence of the insert was confirmed by DNA sequencing. Then, 550 basepair SacII-SacII fragment from pK18-IRES was added to obtain pK18-IRES-(rtTA2^S-M2). The sequence of the The IRES-(rtTA2^S-M2) module of the tet activator plasmid, pK18-IRES-(rtTA2^S-M2) was confirmed by DNA sequencing.

To show that the vectors were functionally active, these were transfected alone or together into 293 cells (Figure 3). Cells were cultured in serum-free media without or with various doses of doxycycline. At 44 hr post-transfection, total RNA from transfected cells was isolated for qRT-PCR. Culture media were concentrated for Western blot analysis of Lefty. Activator was expressed only in cells transfected with the activator vector (Figure 3A) Transfection with *Lefty* or dose of Dox did not alter the expression of the activator. On the other hand, Lefty was expressed only when both activator and *Lefty* were transfected into cells (Figure 3B). Moreover, the expression of *Lefty* was dependent on the dose of Dox present in the culture medium and was induced significantly up to 50 fold (more than three orders of magnitude). *Lefty* expression was induced in a dose ragne of 10-1000 ng/ml with *Lefty* slightly decreasing in medium with 6000 ng/ml of Dox. According to Clontech, such a degree of responsiveness to Dox in transient transfection should allow Dox-dependent upregulation of *Lefty* expression in clonal cell population and in in transgenic animals by about 1000 fold.

Western blot analysis of culture media also showed Dox dose-dependent increase of Lefty (Figure 3C). In the absence of Dox, some basal level of *Lefty* was found in the medium. However, in presence of Dox, *Lefty* increased in the medium at a dose range of 10-1000 ng/ml. Consistent with results from qPCR, *Lefty* was lower in medium with 6000 as compared with 1000 ng/ml of Dox.

Lefty is expressed in stromal cells that undergo decidual differentiation (7). To show regulated expression in cells poised to express *Lefty*, stromal fibroblasts were isolated from placental membranes and were transfected with *Lefty*, rtTA or both in the absence or presence of various concentration of Dox (Figure 4). qPCR of RNA isolated from these cells, 48 hr after transfection, showed that *Lefty* was expressed when cells were transfected with both *Lefty* and rtTA. *Lefty* increased in a dose-dependent fashion with increasing doses of Dox present in the medium. A low basal level of expression of *Lefty* is likely due to the ability of these cells to endogenously make *Lefty*. Western blot analysis of the cell lysates confirmed qPCR

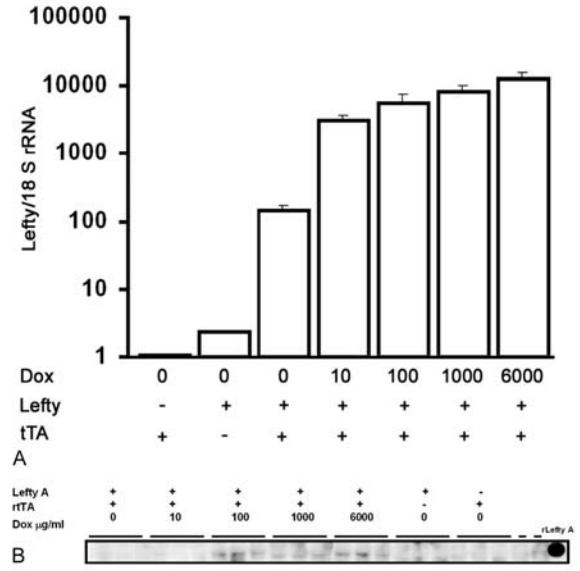

Figure 4. Testing the *Lefty* and rtTA constructs in human decidual fibroblasts. Cells in 60 mm plates were transfected, incubated in serum-free media with various doses of doxycycline (Dox) and harvested by trypsinization 44 hr post-transfection. A. Samples were divided for total RNA isolation and protein sample preparation, which were subjected to quantitative RT-PCR (A) and Western blot analysis for *Lefty* (B). Values were expressed as means±SD.

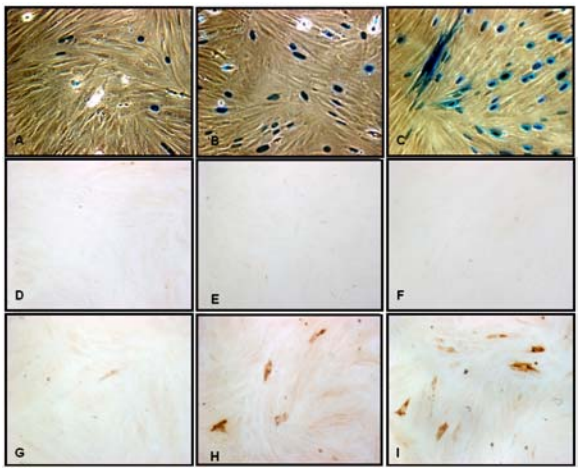

Figure 5. Particle-dependent transduction of β gal and *Lefty* with adenoviral vectors. Decidual cells were isolated from a third trimester placenta by collagenase P and were passaged two times. The cells of the third passage, were transduced with 100 (A), 500 (B) and 2500 (C) β gal adenoviral (Ad-β-gal) and *Lefty* (Ad-*Lefty*) adenoviral particles/cell. 48 hr later, cells were harvested and stained with X-gal (duration of staining, 3 hr) Decidual cells were also transduced with 500 (D), and 2500 (E) empty vector (Ad-CMV) and with 0 (F), 100 (G), 500 (H) and 2500 (I) particle/cell *Lefty* (Ad-*Lefty*) adenoviral particles. 48 hr later, cells were harvested and immunostained with antibody to *Lefty*. Original magification: X40.

findings. When cells were transfected with both *Lefty* and rtTA, *Lefty* became detectable in presence of 100-6000 ng/ml of Dox. *Lefty* was undetectable in the medium in the absence of Dox or when cells were not transfected with *Lefty*, rtTA or both.

4.2. Construction and preparation of Ad-*Lefty* recombinant virus

We used Ad5 E1-replacement virus to generate an Ad-*Lefty* recombinant virus (Ad-*Lefty*). The coding sequence of *Lefty* was inserted into the Ad transfer vector, pAd-CMV, as described in Evans *et al*, (8). This plasmid contains the left 450 bp of Ad5, a CMV promoter/enhancer, cloning sites, followed by Ad5 sequences from 3330-5790. Splicing and polyadenoylation signals are provided by the Ad5 E1B region. The pAd-CMV-*Lefty* transfer plasmid was built into the recombinant Adenovirus following the method of Stow *et al* (9). Recombinant virus was plaque purified, amplified on 293 cells and confirmed by DNA sequencing. Virus particles were purified by two successive rounds of cesium chloride equilibrium centrifugation and quantified by Absorbance at 260 nm where 1 O.D. = 1 x 10^{12} virus particles/ml. All manipulations for the construction of virus were performed as described (10).

To determine whether induction of genes by adenovirus was dependent on the number of viral particles, decidual cells were transduced with various numbers of viral particles (Figure 5). Decidual cells were isolated from a third trimester placenta by collagenase P and were passaged two times. The cells of the third passage, were transduced with the adenovirus Ad-*Lefty*, Ad-β-galactosidase (Ad-β-gal) or empty vector (Ad-CMV). The number of cells positive for β-gal was directly dependent on the number of viral particles in the range of 100-2500 particles/cell. When transduced with Ad-*Lefty*, the intensity and the number of cells immunoreactive for *Lefty* was also directly proportional to the number of viral particles (Figure 5). 48 hours after transduction, cells were harvested. Lysates from cells were subjected to Western blot analysis for *Lefty* (Figure 6).The precursor and processed forms of *Lefty* were present, in a particle number-dependent manner, in the cell lysate of cells transduced with Ad-*Lefty* and not in ad-CMV.

In summary, we have constructed two model systems for regulated expression of *Lefty*. This set of vectors have the advantage that they can be used for defining the role of *Lefty in vitro* in cell lines or primary cultures and also should be suitable for *in vivo* studies. Because *Lefty* knockout was embryo-lethal, overexpression of *Lefty* by transgenic approach might also cause embryo lethality (11). Thus, the tet-on constructs should allow production of conditional *Lefty* transgenic mice for *in vivo* characterization of *Lefty* function during development and adulthood. *Lefty* is useful in the treatment of cancer and fibrotic disorders. For this reason, the Ad-*Lefty* might be useful in gene therapy internventions.

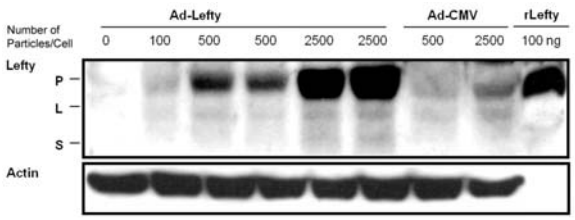

Figure 6. Decidual cells were isolated from a third trimester placenta by collagenase P and were passaged two times. The cells of the third passage, were transduced with the adenovirus encoding *Lefty* (Ad-*Lefty*) or with empty vector (Ad-CMV). 24 hours after the transduction, the culture medium was changed to serum free medium and the cells and their media were collected, 24 hr later. Lysates from cells (A) and their media (B) were subjected to Western blot analysis for *Lefty*.The precursor form of *Lefty* (P) and processed forms of lefty (L: long form, S: Short form) was present in the cell lysate of cells transduced with Ad-*Lefty* in a viral particle number-dependent manner and was absent in the lysates of cells transduced with ad-CMV. rLefty: recombinant lefty used as control.

5. ACKNOWLEDGEMENT

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Key Words: *Lefty*, Ebaf, *In vitro*, *In vivo* Cell ine, Adenovirus, Tetracycline, Transfection, Transductioin, Regulation

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