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14. ABSTRACT The Transforming Growth Factor beta (TGF-) superfamily includes three isoforms designated TGF-1, 2 and 3. All three isoforms are secreted as latent complex where the TGF- cytokine is non-covalently associated with an isoform specific latency-associated peptide (LAP). Mature cytokine binds cell surface receptors only after release from its LAP making extracellular activation a critical regulatory point for TGF-bioavailability. Proposed activation mechanisms include proteolysis and conformational changes. Previous work from our laboratory showed that latent TGF-1 (LTGF-1) is efficiently activated upon exposure to reactive oxygen species (ROS). ROS activation is restricted to the LTGF-1 isoform. Because of the amino acid sequence differences between the three LAPs, we postulate that the specificity of this activation mechanism lies within the LAP. Furthermore, we hypothesize that the presence of a metal in the latent complex could provide a redox active center for this process. Redox mediated activation provides a novel mechanism for TGF- participation in tissues undergoing oxidative stress. Moreover, this would allow TGF-1 to act both as a sensor of oxidative stress within tissues as well as a transducer of that signal by binding to its cellular receptors.					
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Introduction

The Transforming Growth Factor beta (TGF- β) superfamily includes three isoforms designated TGF- β 1, β 2 and β 3. All three isoforms are secreted as latent complex where the TGF- β cytokine is non-covalently associated with an isoform specific latency-associated peptide (LAP). Mature cytokine binds cell surface receptors only after release from its LAP making extracellular activation a critical regulatory point for TGF- β bioavailability. Proposed activation mechanisms include proteolysis and conformational changes. Previous work from our laboratory showed that latent TGF- β 1 (LTGF- β 1) is efficiently activated upon exposure to reactive oxygen species (ROS).

ROS activation is restricted to the LTGF- β 1 isoform. Because of the amino acid sequence differences between the three LAPs, we postulate that the specificity of this activation mechanism lies within the LAP. Furthermore, we hypothesize that the presence of a metal in the latent complex could provide a redox active center for this process. Redox mediated activation provides a novel mechanism for TGF- β participation in tissues undergoing oxidative stress. Moreover, this would allow TGF- β 1 to act both as a sensor of oxidative stress within tissues as well as a transducer of that signal by binding to its cellular receptors.

Body

Specific Aims

1. Characterize the interaction between reactive oxygen and latent TGF- β
2. To identify and localize the redox-metal center within the latent TGF- β
 - a. We have begun to optimize a method based on chemiluminescence and colorimetric detection assays to identify the presence of metal cofactors.
 - b. We initiated the generation of an immortalized TGF- β null mouse embryonic fibroblast (MEF) line that will be used in transfection studies aimed at expressing either wild type LTGF- β 1 or LTGF- β 1 mutants sensitive or not to ROS.
3. Determine the three-dimensional structure of latent TGF- β

Progress

Because Aim #1 is completed, to date, the efforts have been centered into Aim #2. For the *Aim #2 section a*, all the methodology for the chemiluminescent detection of LTGF- β have been set up as described in the last report but we do not have the sufficient concentration of LTGF- β to be tested. For the *Aim #2 section b*, two cell lines of immortalized mouse embryonic fibroblast (MEF) have been obtained. One of the MEF cell lines is a wild type genotype meanwhile the second is knockout for TGF- β . These mutants are being used for transfection studies to express either the wild type LTGF- β 1 or LTGF- β 1 mutants sensitive or not to ROS. About Aim #3, the crystal structure of LTGF- β is being determined by Dr. Peter Walian.

Aim 2. To identify and localize the redox-metal center within the latent TGF- β (LTGF- β)

To further study the activation of LTGF- β 1 by ROS, we will transfect immortalized wild type or TGF β -null MEF with cDNAs encoding wild type LTGF- β 1 or a mutant LTGF- β 1 bearing a point mutation that substitutes methionine for alanine at position 253 (Jobling et al. 2006). We have shown that this M253A mutation leads to loss of response of LTGF- β 1 to ROS. Once the cells will be successfully transfected an experiment will be done to test the effect of IR related to ROS generation.

Because transfection efficiency is very low for primary fibroblasts and because stable transfection is a long process, we propose to establish immortalized MEF cell lines prior to transfection. Primary MEF grow rapidly and are healthy until the 3rd passage, then they grow slowly and die in large number due to senescence. However, MEF cultures maintained for more than 20 passages, around 40 to 50 days, undergo spontaneous immortalization (Parrinello, 2003). We followed a recently optimized immortalization procedure (Jianming Xu, 2005). The first step consisted of maintaining in culture MEFs isolated from wild type and TGF- β null mouse embryos until they reached immortalization. We successfully obtained immortalized MEFs after 22 passages for the wild type and 24 passages for the TFG- β null. Once immortalized, these two MEF cell lines have been expanded, and actually they are being subjected to transfection.

During the immortalization process of a cell line, some mutations are acquired by the cells. These alterations produce changes into the cell morphology and growth rate. Along the immortalization process of both MEF cell lines two main changes on their growth rate and morphology have been noticed (Figure 1 and 2). Once immortalized the WT MEF present a round shape while the TGF β Null are fusiform. Also the growth rate increased as expected for

immortalized cells. Although, the TGF β Null cell line presents a 5 fold growth rate when compared to WT MEF cell line. One of the functions of the TGF β is the cell growth regulation, this suggest that the lack of TGF β production by the Null cell line could increase the cell growth rate. Once the both MEF cell lines had been immortalized we proceeded with the transfections.

Three mutant versions of LTGF β -1, bearing methionine $\rightarrow$ alanine substitutions at three different positions (M112A, M132A or M253A, respectively) have been cloned into the mammalian cell expression vector pCDNA3 as described before (Jobling et al. 2006). Because the pCDNA3 vector is not suitable for retroviral gene transfer technology our first approach was based on the transfection by lipofection. The pCDNA3 vector uses neomycin (G418) as a selection method. We performed a cell death curve for both MEFs cell lines to establish the minimal concentration of antibiotic necessary to kill non transfected cells. The MEF cell lines were tested for increasing concentrations of G418. The results for the cell death curve showed that the MEF WT cell line is sensitive to neomycin (400 μ g/mL), and surprisingly the MEF TGF β -null was resistant for all the concentrations. This result indicates that a stable transfection of the TGF β -null cell line is impossible with this vector and actual strategy.

A second strategy was performed using two vectors simultaneously, the vector of interest (pCDNA3 containing the cDNAs encoding for wild type LTGF β -1 or a mutant LTGF β -1 and a second vector (pPUR) which give a puromycin resistance. Both vectors were transfected at a 10:1 ratio respectively. The transfection method was lipofection, using lipofectamine 2000 (invitrogen) according the manufacture's guidelines. The cell lines were selected with puromycin (Figure 3). Due to the low number of transfected cells they were not able to grow and expand. Besides that we didn't obtained enough transfected cells, the cells generated are a heterogeneous population. The most part of the population of transfected cells corresponds to the ones that incorporated both vectors, expressing our cDNAs of interest, but a lower percentage of cells only incorporate the pPUR vector which give resistance to puromycin but don't express the cDNAs of interest. Due to the low transfection efficiency and the added cell population heterogeneity of the transfected cells we decided to use a different approach to transfect the MEF cell lines.

A third strategy, currently in progress, was designed to perform a stable transfection of the LTGF β -1 and the three mutant versions (M112A, M132A or M253A). In this case we first cloned and purified the pCDNA3 containing our cDNAs of interest. Then the vector was digested with the restriction enzyme EcoRI to cut our cDNA of interest from the pCDNA3 vector. The cDNAs were purified from the vector using a high grade agarose gel. Finally we ligated the cDNAs with the vector pMSCVpuro. Actually we generated satisfactory four pMSCVpuro vectors each one containing the LTGF β -1 or one of the mutant versions. We will transfect each one of this four pMSCVpuro vectors into PT67 cells using lipofectamin 2000. These cells will be selected with puromycin. The transfected PT67 cells will start producing viral particles containing our sequences of interest. Once selected the PT67 cells will be grown for 48-72h in order to generate enough concentration of viral particles. The viral particles are secreted to the culture media which will be collected. The culture media containing the viral particles will be incubated with our MEF cell lines (MEF WT and MEF TGF β -null) to transduce them. Then a puromycin selection will be performed to select the transduced MEF cells.

After the selection with puromycin the transduced MEF cells will be switched to serum-free conditioned medium. Media from MEF cultures will be collected and treated with ascorbic acid or Fe(III) to determine ROS activation. Medium subjected to heat, a treatment known to result in LTGF β -1 activation, will be used as a positive control.

A second set of transduced cells for both MEF lines will be treated with ionizing radiation (IR) at 1Gy. Both transduced MEF cell lines will be maintained with serum replacement media for four days after IR irradiation. Four days after irradiation the media will be collected to measure the LTGF-1 as described before. A portion of the cells will be stained with alpha-smooth muscle actin (α -SMA) to evaluate any morphological changes due to IR and/or the effect of the mutant LTGF-1.

Aim 3. To determine the three-dimensional structure of latent TGF- β

Prior collaborations with Dr. Peter Walian here at LBNL, have not yielded conditions for crystallization of LTGF- β .

Key Research Accomplishments

- Development of immortalized wild type and TGF- β null MEF cell lines.
- Generation of a new vector capable of retroviral gene transfer technology with cDNAs encoding wild type or three mutant versions of LTGF-1 (M112A, M132A, M253A). These vector will be used to generate viral particles that will transducer the previously immortalized MEF cell lines

Conclusions

The expression of LTGF- β 1 mutant constructs into MEF will provide further mechanistic understanding of ROS-mediated TGF- β activation opening a door to a new set of *in vitro* experiments related with IR, ROS and TGF- β .

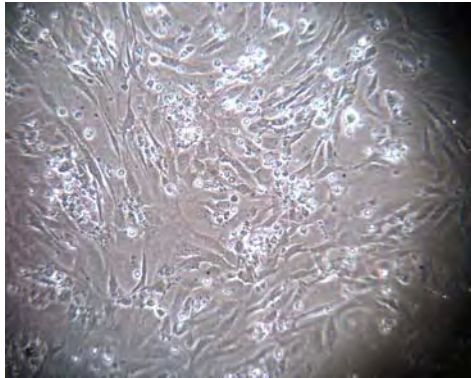
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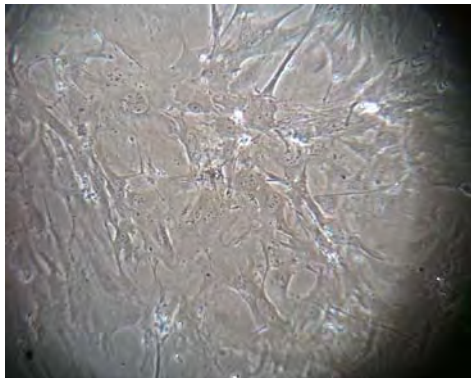
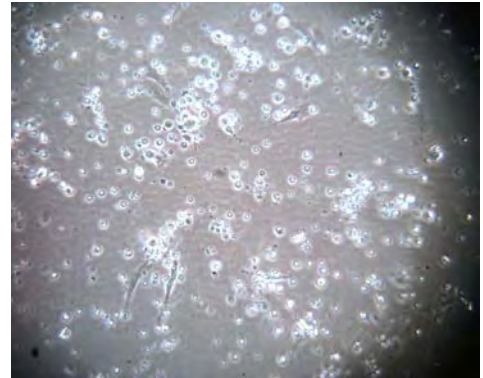
Supporting data

MEF

MEF TGF- β



Passage 1



Passage 2



Passage 3



Figure1: During the firsts passages both cell lines have similar morphology but the TGF β null show a slower cell division rate. Around the tenth passage the morphology of the cells start to change. The WT MEF show a flat and round shape meanwhile the Null are more thin and elongated.

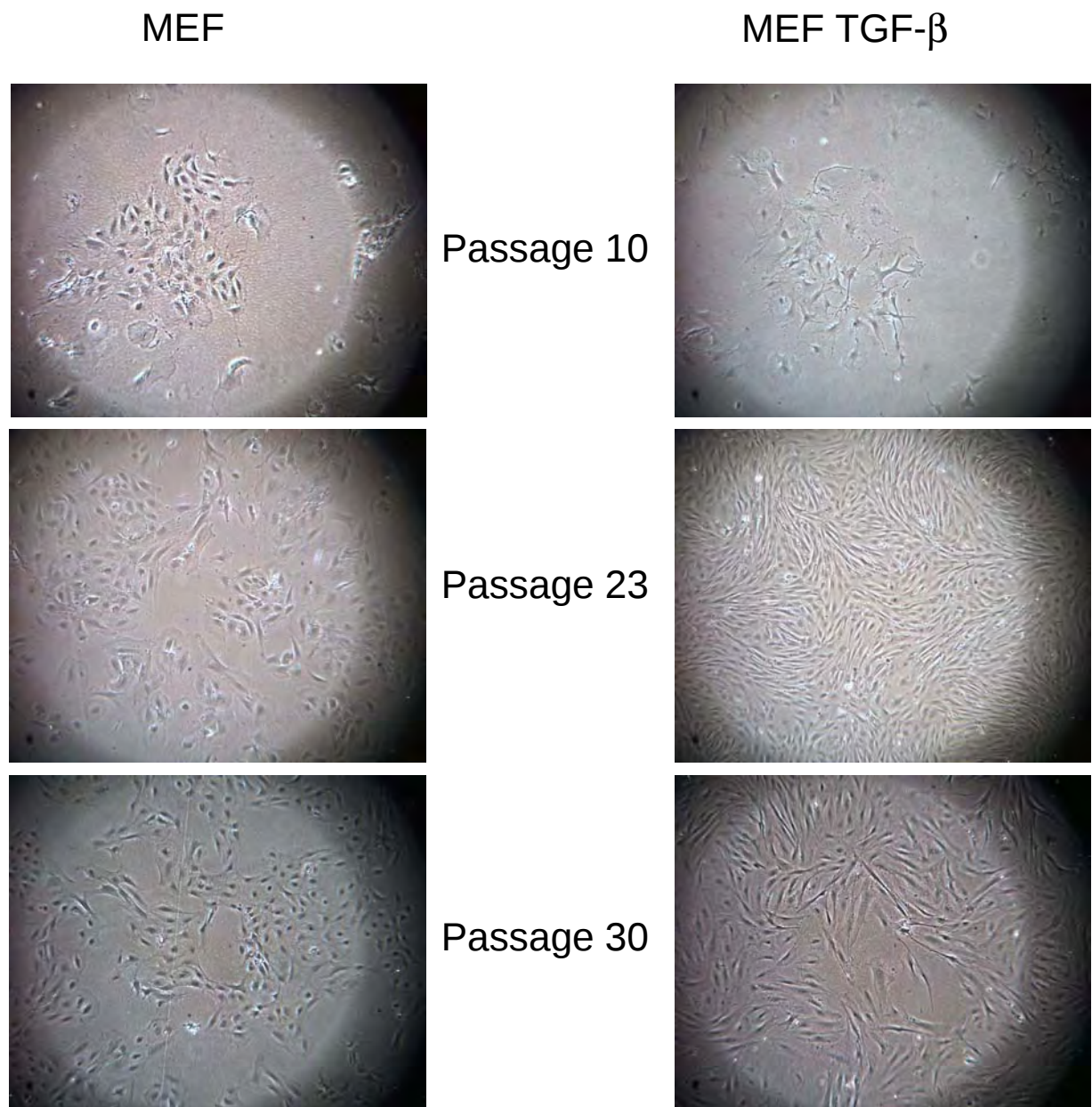
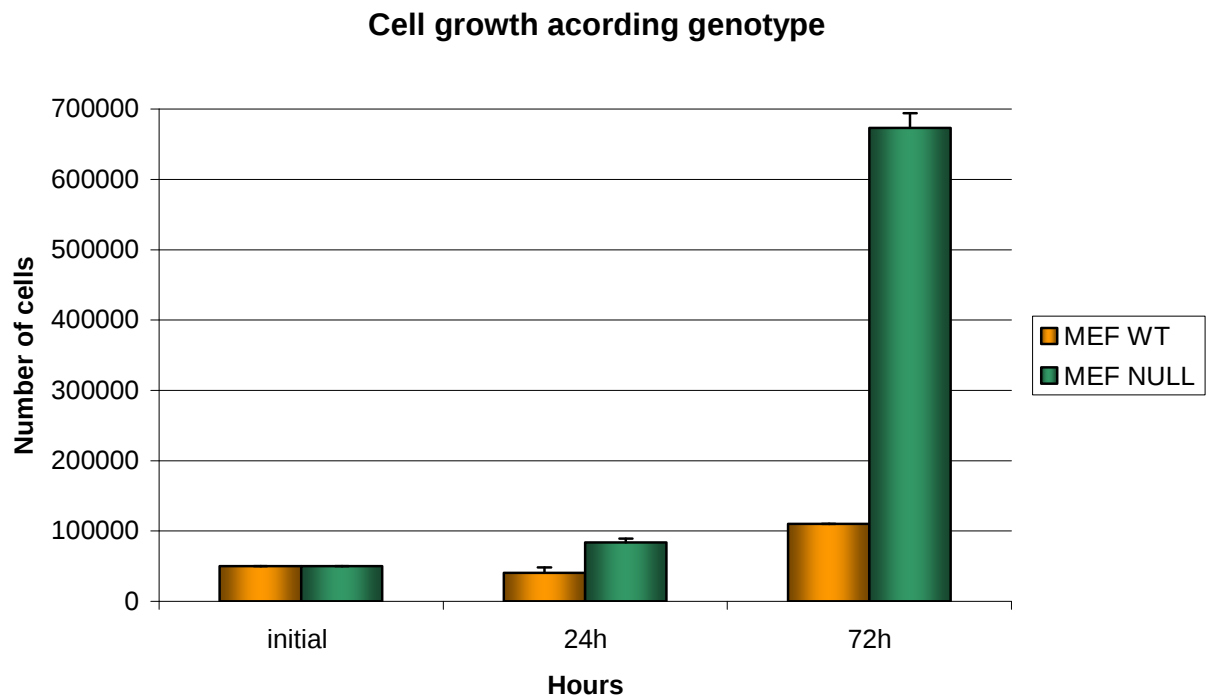


Figure2: After passage 20 both types of cells increased the growth rate. MEF WT presents a round morphology and the TGF β null have a fusiform shape. After passage 24 the Null cell line increased their growth rate five fold compared to the WT.



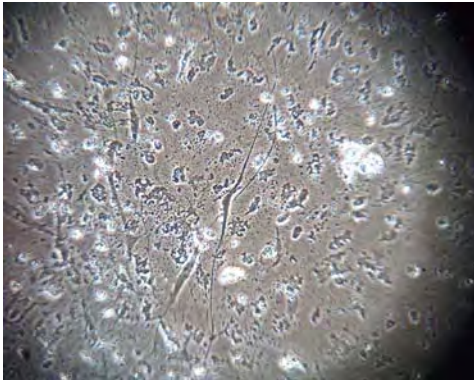
Graph 1: Cell growth curve. After immortalization both cell lines MEF WT and MEF TGF β Null increased their growth rate. Although, the TGF β knockout genotype shows a 5 to 6 fold growth rate than the wild type.

MEF WT

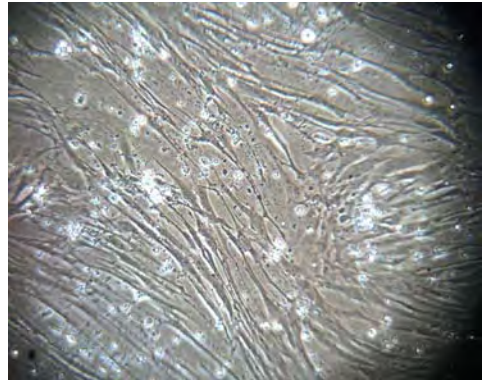


Selection:
Day 1

MEF TGF- β null



Selection:
Day 3



Selection:
Day 6



Figure3: Puromycin selection. After a week selecting with puromycin, a low number of transfected cells were present.