

## High Temporal Resolution of ERK Activity in Response to Mechano-chemical Stimuli

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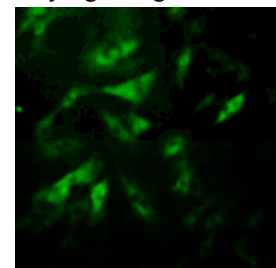
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**Statement of Purpose:** The potential of stem cell therapy in treating vascular diseases such as hypertension are immense and mesenchymal stem cells (MSCs) are particularly feasible candidates. Consistent and rapid differentiation of MSCs into mature and functional vascular phenotypes is essential to realizing this therapeutic potential. While protocols exist for differentiating MSCs toward smooth muscle cell (SMC) phenotypes, such methodologies are complex and often time consuming, utilizing multiple factors and requiring weeks to reach maturity. Recently, we reported that specific substrate elasticity and geometry can induce early SMC markers within 24 hours (Wingate K. *Acta Biomater.* 2012;8:1440-1449). By resolving the spatiotemporal mechanisms of initial differentiation cues and processes we may establish valuable insights towards early signaling events, reducing regimen duration, and optimizing material design for MSC differentiation. Extracellular signal-regulated kinase (ERK) has been implicated in regulating SMC differentiation from MSCs and the sub-48 hour temporal dynamics of the relationship between mechano-chemical differentiation cues and ERK response is relatively unexplored. To design a system that allows observation of real-time responses to various mechano-chemical stimuli, we have transfected the highly sensitive fluorescence resonance energy transfer (FRET)-based biosensor NES-EKAR (Harvey CD. *Proc. Natl. Acad. Sci. U.S.A.* 2011;105:19264–19269) that measures cytoplasmic ERK activity into rat MSCs. After successful transfection, the sensor's ability to measure ERK activity in response to mechanical and chemical stimuli was validated.

**Methods:** Rat MSCs (Lonza Group Ltd, Switzerland) were seeded and cultured, and maintained in Dulbeccos Modified Eagles Media (DMEM) (Hyclone, Logan/UT), with 10% defined FBS for MSCs and 1% Penn/Strep (Invitrogen, Carlsbad, CA) at 37°C and 5% CO<sub>2</sub>. Cells were transfected with NES-EKAR plasmid donated by Dr. Xuedong Liu, University of Colorado, using Lipofectamine 2000 (Life Technologies, Grand Island, NY). Transfected cells were selected with Blasticidin S (Invivogen, San Diego, CA) treatment of 5ug/ml for 24 hours. Transfected cells were imaged using a Zeiss Axiovert 40 CFL microscope (Carl Zeiss Microscopy, Thornwood, NY) with a FITC filter to confirm transfection of plasmid. Sensor activity was imaged with ImageXpress microXL microscope (Molecular Devices, Sunnyvale, CA) with the MetaXpress software suite and using Chroma filters T4551p and ET535/30m (Chroma Technology Corporation, Bellows Falls, VT) for ECFP and ECFP/EYFP FRET respectively. A prospective study was utilized to preliminarily validate sensor response to mechanical and chemical stimuli. Cells were imaged every 5 min for 1 hour at 37°C and 5% CO<sub>2</sub>. After 4 min,

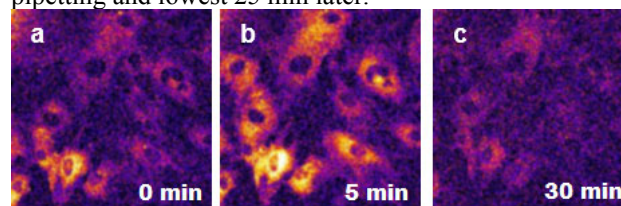
MEK inhibitor U0126 (Promega, Madison WI) was pipetted in for a final concentration of 50uM, which also acting as a mechanical stimulus by agitating the media. Raw images were processed and background corrected using ImageJ software to yield FRET/CFP images.

**Results:** We were able to successfully transfect and select for rat MSCs expressing the cytoplasmic ERK sensor, seen in Figure 1. By inducing mechanical agitation of the culture media followed by inhibition of



**Figure 1. Rat MSCs transfected with NES-EKAR**

MEK by U0126, we validated the functionality of the ERK sensor. The FRET/CFP background-corrected images of transfected rat MSCs display distinct differences in intensity, where the brighter the pixel, the greater the FRET ratio and thusly, the greater the activity of ERK. Fig 2a is of ERK activity before the pipetting of U0126 at time 0. Fig. 2b shows an increase of ERK activity at 5 min, 1 min after pipetting. Fig. 2c indicates that 25 min later, ERK activity had dropped to below levels of activity at time 0. The mean intensity of the pixels in each image of these results follow this trend (not shown), with the FRET signal being the highest just after pipetting and lowest 25 min later.



**Figure 2. FRET/CFP images of rat MSCs**

**Conclusions:** These results show agitation of culture media can mechanically stimulate ERK activity, while the MEK inhibitor directly quenches this increase in activity, indicating that the NES-EKAR ERK biosensor can be transfected into rat MSCs and retain functionality. We are creating a dynamic temporal ERK profile of rat MSC responses to differentiation cues such as substrate elasticity/geometry and morphogens to be correlated with vascular differentiation of MSCs.

**References:** Wingate K, Bonani W, Tan Y, Bryant S, Tan W. 2012. Compressive elasticity of threedimensional nanofiber matrix directs mesenchymal stem cell differentiation to vascular cells with endothelial or smooth muscle cell markers. *Acta Biomater* 8, 1440-1449. Harvey CD, Ehrhardt AG, Cellurale C, Zhong H, Yasuda R, Davis RJ, Svoboda K. 2008. A genetically encoded fluorescent sensor of ERK activity. *Proc. Natl. Acad. Sci. U.S.A.* 105:19264–19269