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Development of a novel fluorescent biosensor for dynamic monitoring of metabolic methionine redox status in cells and tissues

Dong Wook Choi [b,l,1], Yeon Jin Roh [a,1], Seahyun Kim [a,1], Hae Min Lee [a], Minseo Kim [a], Donghyuk Shin [c], Jong Ho Park [d], Yongmin Cho [e], Hee Ho Park [f], Yong Sik Ok [g], Donghyun Kang [h], Jin-Hong Kim [h], Lionel Tarrago [i], Nika N. Danial [b], Vadim N. Gladyshev [j], Pil-Ki Min [k,**], Byung Cheon Lee [a,2,*]

[a] Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul, 02841, Republic of Korea
[b] Department of Cancer Biology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, 02215, USA
[c] Department of Systems Biology, Yonsei University, Seoul, 03722, South Korea
[d] Center for Cancer Immunology and Cutaneous Biology Research Center, Department of Dermatology, Center for Cancer Research, Massachusetts General Hospital and Harvard Medical School, Boston, MA, 02114, USA
[e] Department of Systems Biology, Harvard Medical School, Boston, MA, 02115, USA
[f] Department of Biotechnology and Bioengineering, Kangwon National University, 1 Kangwondaehak-gil, Chuncheon-si, Gangwon-do, 24341, Republic of Korea
[g] Korea Biochar Research Center, O-Jeong Eco-Resilience Institute (OJERI) & Division of Environmental Science and Ecological Engineering, Korea University, Seoul, 02841, Republic of Korea
[h] Department of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul, 08826, Republic of Korea
[i] INRAE, Aix Marseille University, BBF, F-13009, Marseille, France
[j] Division of Genetics, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA
[k] Cardiology Division, Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, 06229, Republic of Korea
[l] Department of Biochemistry, College of Natural Sciences, Chungnam National University, Daejeon, 34134, South Korea

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ABSTRACT

Aberrant production of reactive oxygen species (ROS) leads to tissue damage accumulation, which is associated with a myriad of human pathologies. Although several sensors have been developed for ROS quantification, their applications for ROS-related human physiologies and pathologies still remain problematic due to the unstable nature of ROS. Herein, we developed Trx1-cpYFP-fRMsr (TYfR), a genetically-encoded fluorescent biosensor with the remarkable specificity and sensitivity toward fMetRO (free Methionine-R-sulfoxide), allowing for dynamic quantification of physiological levels of fMetRO, a novel indicator of ROS and methionine redox status *in vitro* and *in vivo*. Moreover, using the sensor, we observed a significant fMetRO enrichment in serum from patients with acute coronary syndrome, one of the most severe cardiovascular diseases, which becomes more evident following percutaneous coronary intervention. Collectively, this study proposes that fMetRO is a novel biomarker of tissue damage accumulation in ROS-associated human pathologies, and that TYfR is a promising tool for quantifying fMetRO with potentials in versatile applications.

1. Introduction

Reactive oxygen species (ROS) serve as signaling molecules for numerous biological processes including development, differentiation, and apoptosis. Excess amounts of ROS, however, result in oxidative stress leading to the accumulation of damaged DNA, protein, and lipids (Schieber and Chandel, 2014; Stadtman, 1992). In this context, dysregulated redox homeostasis has been implicated in a myriad of

* Corresponding author. College of Life Sciences and Biotechnology, Korea University, Seoul, 02841, Republic of Korea.
** Corresponding author. Cardiology Division, Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, 06229, Republic of Korea.
E-mail addresses: cardioblues@yuhs.ac (P.-K. Min), cheonii@korea.ac.kr (B.C. Lee).
[1] These authors contributed equally to this work.
[2] College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea.

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pathophysiological and physiological conditions, including cancer, neurodegenerative, cardiovascular diseases, and aging (Choi et al., 2019; Liou and Storz, 2010; Zhou et al., 2018).

Substantial efforts have been made to not only understand the biochemical mechanisms underlying ROS implication in the pathological and physiological conditions, but also to develop biosensors that can measure ROS levels. For example, H_2O_2 levels have been directly monitored *in vivo* and *in vitro* using a H_2O_2 sensor, including HyPer, a genetically-engineered fluorescence protein including circularly permuted yellow fluorescent protein (cpYFP) (Belousov et al., 2006), Orp1 sensor using the bacterial H_2O_2 regulators (Levine et al., 1996; Stadtman et al., 2005), and Prx-RoGFP, a recently developed peroxiredoxin-based redox-sensitive GFP sensor displaying a higher sensitivity compared to the aforementioned sensors, allowing for measuring the metabolic H_2O_2 baseline levels *in vivo*. YFP and GFP, with oxidation on their cysteine residues, have also been exploited to monitor changes in ROS levels *in vivo* and *in vitro* (Hanson et al., 2004; Ostergaard et al., 2001*)*. However, the applications of these sensors for monitoring ROS in various physiological and pathological settings still remain challenging due to the unstable nature of ROS (Niethammer et al., 2009).

Methionine oxidation is a physiological process by which ROS oxidize free methionine (fMet) or protein-based Met (pMet), resulting in the production of methionine sulfoxide (MetO) —diastereomers of MetO, including methionine-R-sulfoxide (MetRO) and methionine-S-sulfoxide (MetSO). Interestingly, such MetO species have recently emerged as a relevant ROS indicator due to its higher stability than the other previously known indicators such as H_2O_2 or oxidized cysteine (Lee and Gladyshev, 2011).

Such MetO has been known to be dynamically regulated by MsrA and MsrB, reducing enzymes only expressed in multicellular system. Mechanistically, MsrA and MsrB reduce MetO to methionine, resulting in the intramolecular disulfide bond formation between cysteine 35 and 37 within the catalytic sites of the reductases. Such oxidized reductases are reversed by thioredoxin (Trx) system, through which the enzyme activities of the reductases are restored. One of the unique properties of the MetO reductases in multicellular system is their differential substrate reactivities (Tarrago et al., 2012) (Tarrago and Gladyshev, 2012). For example, MsrA efficiently reduces both free methionine-S-sulfoxide (fMetSO) and protein-based methionine-S-sulfoxide (pMetSO), whereas MsrB displays a higher sensitivity toward protein-based methionine-R-sulfoxide (pMetRO), but is essentially inactive with fMetRO (Lee and Gladyshev, 2011), leading to an accumulation of fMetRO. Therefore, such fMetRO accumulation, not the other MetO species, led us to hypothesize that fMetRO may serve as a biological marker of not only *ROS per se,* but also tissue damage accumulation following oxidative stress in ROS-associated human pathophysiologies. This diagnostic potential, together with the physiological relevance of methionine redox status in numerous cellular processes including sulfur metabolism, signaling pathways and protein synthesis (Klein Geltink and Pearce, 2019), sparked the scientific interest in developing a biosensor for detecting the physiological level of fMetRO.

Taking advantage of the chemical and biological properties of MetO, genetically-engineered fluorescence-based MetO sensors have recently been developed to quantitatively measure MetO *in vivo* and *in vitro* (Tarrago et al., 2015). These sensors have been created by combining cpYFP with MsrA and MsrB from *Saccharomyces cerevisiae*. However, the various applications of these sensors in monitoring physiological and pathological perturbation of fMetRO have been challenging as they are not substantially sensitive to detect the physiological range of free MetRO concentration (Lee et al., 2008; Tarrago et al., 2015). Such limitations led us to design a new strategy for fMetRO quantification in complex biological samples with a high specificity and sensitivity. To achieve this, we developed Trx1-cpYFP-fRMsr (TYfR), a novel fluorescent biosensor using free methionine-R-sulfoxide reductase (fRMsr), a gene from *E.coli,* which displays a remarkable sensitivity and specificity toward fMetRO, but not fMetSO (Lin et al., 2007a).

Thorough biochemical assessment of TYfR sensor revealed that the TYfR displays a remarkable specificity and sensitivity, allowing for steady states and real-time measurement of physiological concentrations of fMetRO from cells to whole-body levels. More importantly, using the TYfR, we found a significant increase in fMetRO levels in human serum biopsies from patients with acute coronary syndrome (ACS), one of the most severe ROS-related human pathologies, which becomes more evident following percutaneous coronary intervention (PCI). Such observations suggest that our novel platform has the potential to serve as a diagnostically relevant tool for dynamic monitoring of ROS and methionine redox status which represents tissue damage accumulation in ROS-associated human pathologies.

2. Results

2.1. Development of the free methionine-R-sulfoxide-detecting biosensor

To develop a sensor for detecting a physiological level of fMetRO, *fRMsr* was cloned into an expression vector with mutant *Thioredoxin 1* (*Trx1*), in which the resolving cysteine was replaced with serine, Cys35Ser (Fig. 1). Of note, unlike MsrB in multicellular system, fRMsr efficiently and specifically reduces fMetRO, but not fMetSO, through the unique coordination between two resolving cysteine residues (Cys84 and Cys94) located in the catalytic sites of the fRMsr and, Trx system (Etienne et al., 2003; Le et al., 2009; Lin et al., 2007a). Then, several circular permutated fluorescent protein genes, *cpBFP*, *cpYFP*, and *cpRFP*, were inserted between *fRMsr* and *Trx1*. Given the natural transient disulfide bond between fRMsr and Trx1, we speculated that fMetRO treatment will lead to this intramolecular structural change also when the proteins are fused, thereby changing the fluorescence spectra of fluorescent proteins that links these oxidoreductases. Interestingly, the fusion protein with cpYFP, displayed a specific change in fluorescence spectra upon treatment with 10 mM fMetRO (Fig. 1A and B), whereas those made with cpBFP or cpRFP were not reactive (Figure S1A-F). We named the sensor created with the cpYFP, 'TYfR' (Fig. 1A and B). For the spectrophotometric characterization of this sensor, we produced the recombinant protein and reduced it with glutathione (GSH). The spectrophotometric data of reduced TYfR revealed that it displayed two peaks for absorbance and excitation with maxima at 430 nm and 500 nm and one peak for emission at 535 nm (Fig. 1C and D). fMetRO treatment altered the excitation and emission spectra of TYfR; the fluorescence intensity of the emission peak increased at 535 nm, while that of the excitation peak increased at 500 nm, but not at 430 nm, allowing this sensor to be used to detect fMetRO by measuring the ratio of fluorescence intensities (RFI) at the two excitation peaks (500 nm/430 nm, 535 nm for emission) (Figure S2).

2.2. Characterization of the TYfR biosensor

The specificity and sensitivity of the TYfR biosensor were also determined with various substrates. We first found that the RFI of TYfR did not change in the presence of hydrogen peroxide (H_2O_2), suggesting that TYfR specifically monitors fMetO, but not oxidative stress *per se* (Fig. 2A, Figure S2A). This notion is further supported by data in Fig. 2B, wherein NaOCl, a strong oxidizing reagent, only changes the RFI of TYfR following incubation with Met for MetO production. We also measured the RFI of TYfR in the presence of a physiological range of fMetRO (5, 10, or 20 μM)[12] or equivalent concentration of fMetSO. The increase in RFI of TYfR was observed at all the tested concentrations of fMetRO, but not at any concentration of fMetSO, indicating that TYfR displays high substrate specificity and sensitivity (Fig. 2C). Moreover, linear regression analysis showed that the RFI of TYfR was directly proportional to the fMetRO level within the range of fMetRO concentrations tested in Fig. 2B, as well as below the nanomolar concentration (Limit of detection (LOD): 250–500 nM; $R^2 = 0.9882$, $p < 0.0001$) (Fig. 2D and E),

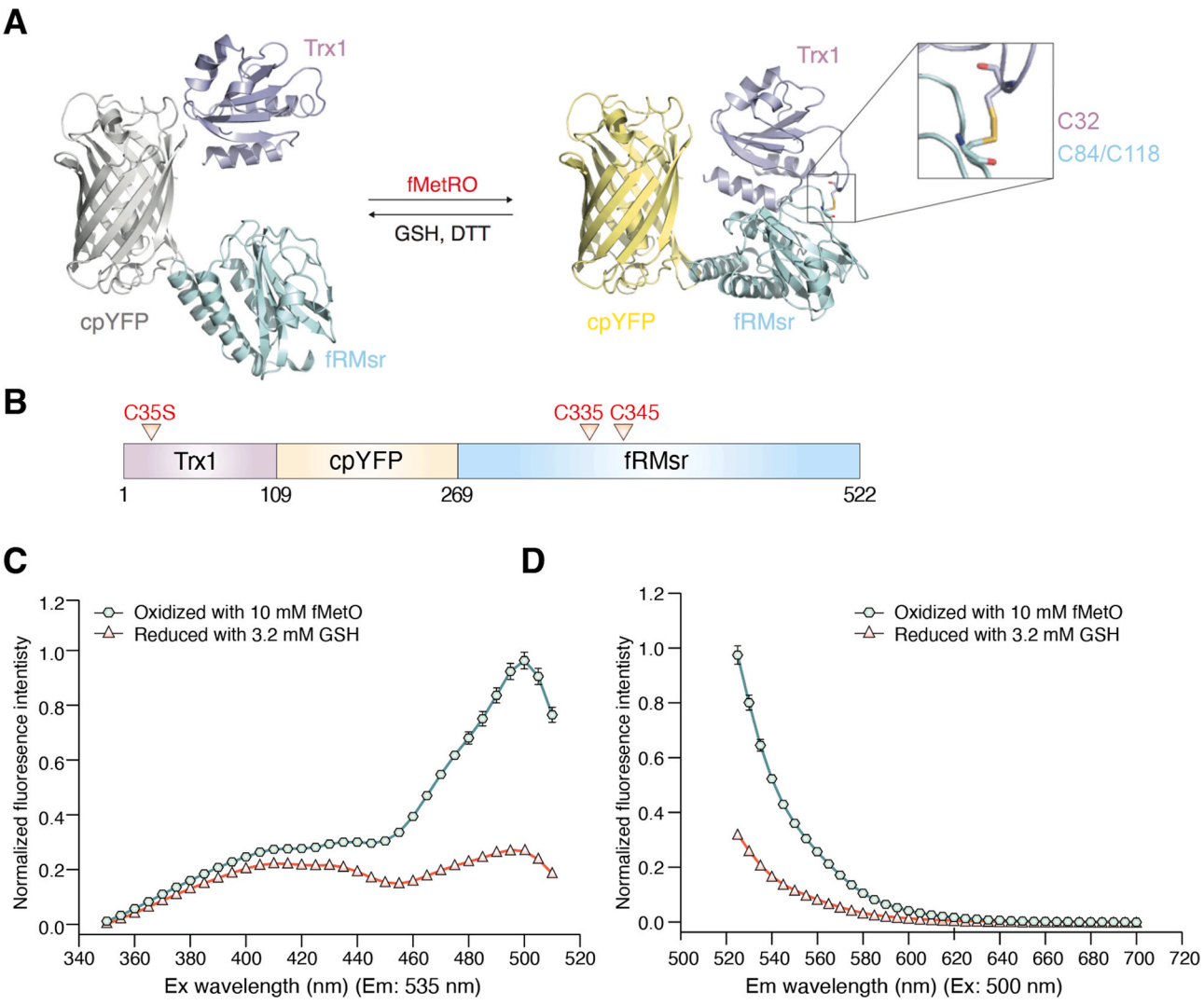

Fig. 1. Development and determination of the spectral characteristics of the TYfR sensor
(A and B) Schematic diagrams of TYfR structure and its potential mechanism of action. *cpYFP* was fused in frame with *fRMsr* and *Trx1*. *Trx1* is a mutant in which the resolving cysteine 35 has been mutated to serine. fRMsr possesses two resolving cysteine residues (Cysteine 66 and 76 equivalent to cysteine 335 and 345 in Trx1/cpYFP/fRMsr plasmid), which may be responsible for fMetRO reduction
(C and D) Excitation and emission spectra of reduced and oxidized TYfR. The sensor was oxidized using 10 mM fMetO or reduced using 3.2 mM GSH, followed by measurement of the fluorescence at the indicated wavelengths. Data have been presented as mean ± SD from three replicate measurements.

suggesting that the TYfR sensor can quantitatively measure physiological concentrations of fMetRO. Of note, MetROx, a pMetRO sensor (Lee et al., 2008), failed to show the linear relationship between RFI and fMetRO concentration in the physiological range (LOD was not measurable in the range tested), although it could still detect the range of pMetRO (Fig. 2F and Figure S2B), indicating that MetROx might not be a relevant biosensor to measure fMetRO level *in vivo*. The observations, taken together with *fRMsr* displaying a high specificity and sensitivity toward fMetRO, led us to propose that TYfR is the first developed sensor for quantification of *in vivo* concentrations of fMetRO.

We then determined the physiochemical properties of TYfR. We first examined the effect of pH on TYfR activity and showed that the maximum dynamic range of the sensor was at a pH of 7.5, substantiating the experiments performed at pH of 7.5 throughout our study, albeit TYfR displayed a higher RFI at pH 8.5 and 9.0 than at any pH conditions tested (Figure S2C-D). We also observed that treatment with dithiothreitol (DTT) (5, 25 or 50 mM) or GSH (3.2 mM) (Figure S2E-F) diminished the RFI of TYfR, which led us to use GSH to generate the reduced form of TYfR.

2.3. *In vivo testing of the TYfR biosensor*

Nanomolar scale sensitivity (250–500 nM) and high specificity of the TYfR sensor led us to examine whether TYfR can detect fMetRO *in vivo*. To achieve this, we transfected a mammalian expression vector encoding TYfR into HEK293FT cells, followed by treatment with 4 mM GSH to maintain the transfected TYfR in the same redox state. Single-cell confocal microscopy coupled with time series analysis was employed to measure dynamic fluorescence changes upon treatment with various substrates. We found that the RFI of TYfR increased upon treatment with 10 mM free methionine-R/S-sulfoxide (fMetO), while GSH treatment led to a decrease in RFI (Fig. 3A and C). Notably, 10 mM H_2O_2 treatment resulted in an increase in the RFI of TYfR (Fig. 3B and D). This observation, together with the results of Fig. 2 A-C, in which TYfR only responded to fMetO produced by oxidative stress, but not to oxidative stress *per se*, suggested that the TYfR sensor can specifically monitor changes in the cellular levels of fMetRO produced by oxidative stress.

Differential oxidative stress status observed across various cellular organelles (Giorgio et al., 2007; Yoboue et al., 2018) led us to hypothesize that the level of fMetRO may also be different from one organelle to

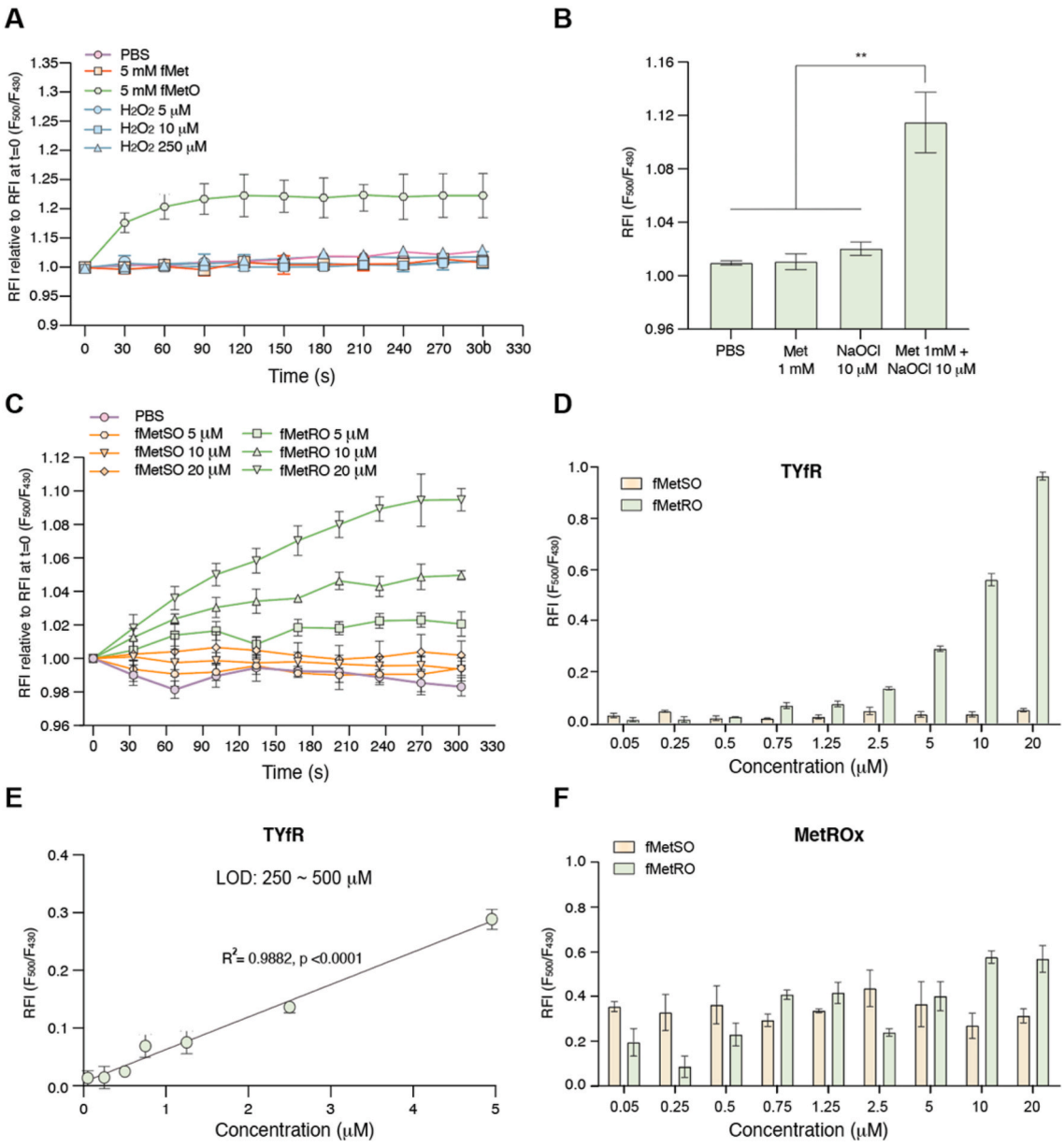

Fig. 2. Specificity and sensitivity of TYfR sensor towards fMetRO

(A and **C)** Kinetics of reactivity of TYfR towards fMet, fMetO, and various concentrations of H_2O_2 **(A)**, fMetRO and fMetSO (5, 10, and 20 μM) **(C)**. Fluorescence variations in $F_{500\ nm}/F_{430\ nm}$ ratios for 5 min were normalized to the value at t = 0 (R_0). Fluorescence excitation intensity ratios $F_{500\ nm}/F_{430\ nm}$ were measured from spectra recorded at an emission wavelength of 535 nm

(B) Response of TYfR towards Met oxidized using NaOCl. Values of fluorescence intensity at 20 min were normalized to the value at t = 0 (R_0)

(D) Measurement of fluorescence intensities of TYfR at various concentrations of fMetRO and fMetSO. TYfR was incubated with the indicated concentrations of fMetRO or fMetSO. Fluorescence intensities were normalized to fluorescence values of the most oxidized and reduced forms of TYfR within each experiment

(E) Standard curve of fluorescence intensity of TYfR at the fMetRO concentrations tested in **(D)**. LOD = 250–500 nM, $R^2 = 0.9882$ and $p < 0.0001$

(F) Measurement of fluorescence intensity of MetROx at the concentrations of fMetRO and fMetSO tested in (D). MetROx was incubated with the indicated concentrations of fMetRO or fMetSO. Fluorescence intensities were normalized to the fluorescence values of the most oxidized and reduced forms of MetROx within each experiment

Data for all experiments are presented as mean ± SD of three replicates

Abbreviations; Met, methionine; fMetRO, free methionine-R-sulfoxide; fMetSO, free methionine-S-sulfoxide; NaOCl, sodium hypochlorite; H2O2, hydrogen peroxide, LOD: Limit of detection

another. To test this possibility, we cloned vectors encoding TYfR fused with specific peptide sequences that could target TYfR into various cellular compartments including cytosol, nucleus, endoplasmic reticulum (ER), and mitochondrial matrix. As shown in Fig. 3E–F and Figure S3A-D, surprisingly, TYfR displays higher RFIs in the ER and mitochondria, as compared to those in the nucleus and cytosol, suggesting that the fMetRO level may be differentially regulated in these organelles. Taken together, we propose that our TYfR sensor can be used to quantitatively measure the level of fMetRO at subcellular levels.

2.4. Detection of endogenous free methionine-R-sulfoxide in serum and lysates

We developed a novel platform through which fMetRO level can be

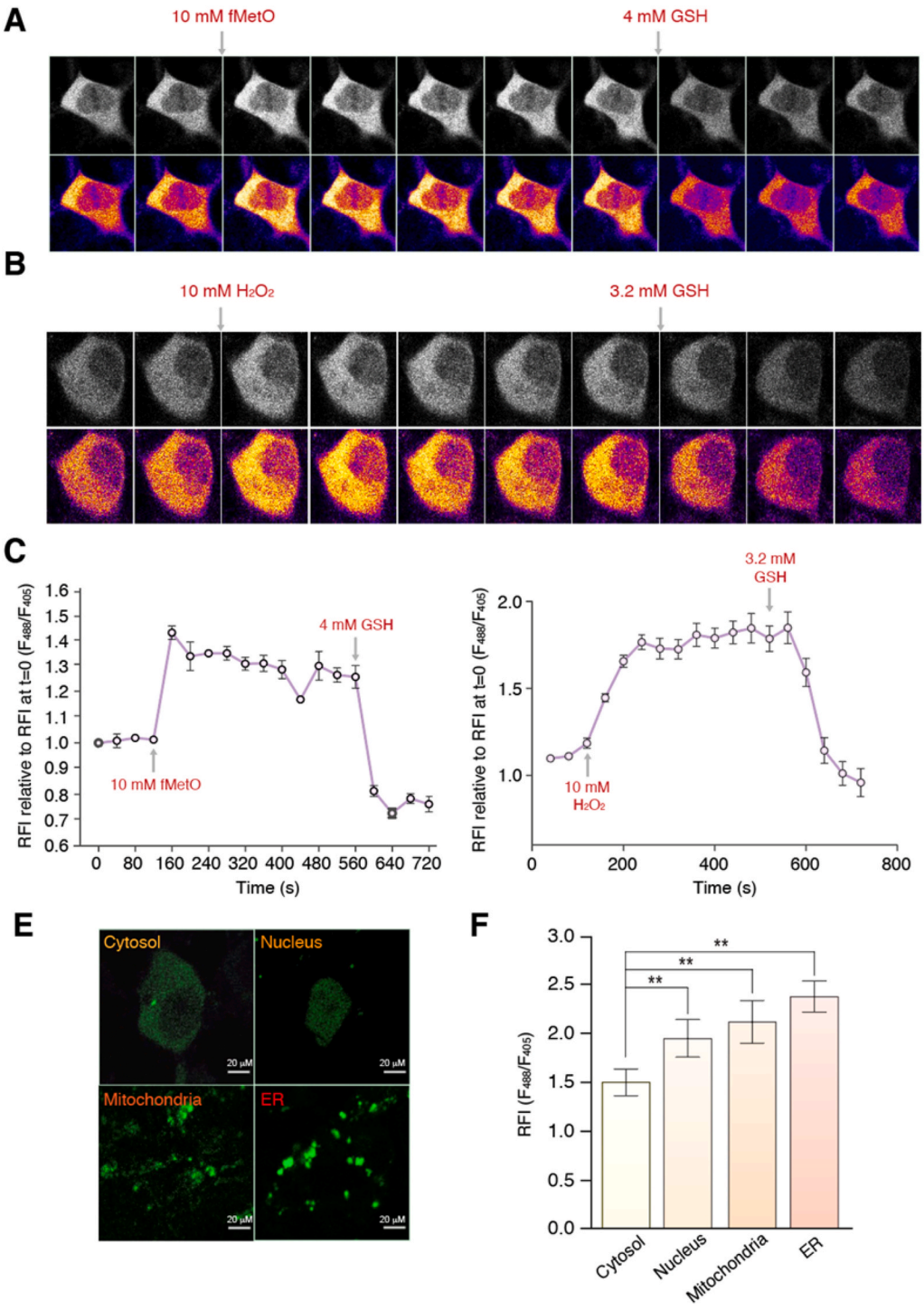

Fig. 3. Measurement of subcellular levels of fMetRO in HEK293 cells treated with oxidizing reagents

(A and C) Time series of raw fluorescent and pseudo-colored images of TYfR in HEK293FT cells before and after sequential addition of 10 mM fMetO and 4 mM GSH **(A)**. The arrows indicate the time point at which the substrates were added. Variation in excitation fluorescence intensity ratios (F_{488}/F_{405}) of TYfR in a time series analysis **(C)**. The ratio is calculated by dividing the background-subtracted fluorescence at 488 nm to the fluorescence at 405 nm. Data have been presented as mean ± SD (n = 4–6)

(B and D) Time series of raw fluorescent and pseudo-colored images of fMetRO sensor in HEK293FT cells treated sequentially with 10 mM H_2O_2 and 3.2 mM GSH **(B)**. The arrows indicate the time point at which the substrates were added. Variation in excitation fluorescence intensity ratios (F_{488}/F_{405}) of TYfR was measured using time series analysis **(D)**. The intensity ratio was calculated by dividing the background-subtracted fluorescence at 488 nm by the fluorescence at 405 nm. Data have been presented as mean ± SD (n = 4–6)

(E and F) Representative images of fluorescence signal from TYfR expressed in different cellular compartments obtained using confocal microscopy **(E)**. Scale bar, 20 μm. The fluorescence intensity ratio (F_{488}/F_{405}) of TYfR was measured in the different compartments **(F)**. Fluorescence was activated at 488 nm and 405 nm. Emission range was >505 nm **p < 0.01 (Bonferroni test). Abbreviations: ER, endoplasmic reticulum; GSH, glutathione.

measured in various types of biological samples (Fig. 4A). Biological samples (e.g., cell lysates and serum from mice) were incubated with the fully reduced TYfR sensor, following their deproteinization to avoid background signal due to macromolecules in the samples. Upon using this platform, we observed a significant increase in RFI, which is proportional to the level of oxidants treated in HEK293FT lysates, suggesting the tool suited for *in vivo* quantification of fMetRO (Fig. 4B and C).

We further investigated whether such a platform is applicable to tissue samples, such as serum from mouse models of ROS production. To this end, we tested the serum from mice challenged with destabilization of medical meniscus (DMM) surgery, which has been implicated in systemic ROS production (Lee et al., 2008; Lepetsos and Papavassiliou, 2016). Notably, DMM surgery led to a significant increase in the serum level of fMetRO, as evidenced by the higher RFI of TYfR incubated with the serum from mice that underwent DMM surgery when compared to the serum from mice that underwent sham surgery (Fig. 4D). More surprisingly, we observed that TYfR sensor becomes fluorescently activated *in vivo* following injection of the TYfR sensor and As_2O_3, a chemical that induces oxidative stress (in the form of H_2O_2) leading to systemic ROS production and tissue damage (Hu et al., 2020), suggesting that TYfR can also be employed for real-time measurement of *in vivo* fMetRO (Fig. 4E and F). In addition, TYfR also detected the accumulation of fMetRO in the serum extracted from As_2O_3-treated mice over a time period of 12 h (Fig. 4G). Such observations suggest that fMetRO may be a relevant indicator of the dynamic methionine redox status which represents tissue damage accumulation following oxidative stress. These findings, taken together with our powerful platform, affirmed the potential use of TYfR for measuring damage accumulation following oxidative stress in the context of ROS-related human pathologies.

2.5. Clinical implication of TYfR use to measure oxidative stress during myocardial ischemia-reperfusion injury

A massive burst of ROS is a pathophysiological consequence of restored blood flow following PCI for treating ACS including myocardial infarction and unstable angina (Granger and Kvietys, 2015) (Fig. 5A), leading to severe tissue damage near the surgical regions.

We tested whether TYfR can be employed as a relevant tool for measuring the accumulated tissue damage following increased oxidative stress following PCI. For this, the levels of fMetRO were measured in human serum biopsies from 1) control subjects having chest pain, but with normal coronary arteries (control, n = 8), and 2) ACS patients (n = 9) before and after PCI (Fig. 5A and B). First, we observed whether there was significant increase in fMetRO levels in serum from ACS patients as compared to those in the serum from control subjects (Fig. 5B), confirming the well-established correlation between oxidative stress and ACS incidence. More importantly, across all the patients tested, fMetRO levels were significantly higher in the serum collected a day after PCI ($p = 0.0055$) (Fig. 5C); this is consistent with a previous report showing that the serum ROS level in a canine model of PCI remained high for a day after PCI (Bolli et al., 1989). Such an observation in the human biopsies from ACS patients undergoing PCI highlighted the potential use of fMetRO as a novel biomarker for accumulated tissue damage following oxidative stress in ROS-associated human pathophysiology.

3. Discussion

fMetRO has recently been proposed as a biological indicator of ROS, owing to its better stability than the other known ROS indicators including such as H_2O_2, cysteine, and sulfenic acid (Claiborne et al., 1993). For example, a significant accumulation of fMetRO levels has been observed in the serum from mice challenged to various forms of oxidative stress, in aged mice and mice fed on a selenium-deficient diet (Lee et al., 2008). However, physiological and pathological relevance of fMetRO in various ROS-related physiologies and pathologies have yet to be explored, due to the lack of a sensor for the detection of fMetRO concentration in the physiological range. For example, 1) high performance liquid chromatography (HPLC) method, which has been previously used to measure fMetRO (Lee et al., 2008), was able to detect the range of 5 μM–500 μM concentration of fMetRO (Figure S4A) although it failed to detect either nM concentration of fMetRO *in vitro* or more complex biological samples such as fMetRO in serum biopsies (Figure S4B). 2) Gas chromatography-mass spectrometry (GC/MS-MS) has been shown to detect fMetO with a high sensitivity following a specific derivatization method (Sochaski et al., 2001) (Figure S4C–F). However, the caveat of the method is that it cannot distinguish fMetRO from fMetSO, not allowing for a specific detection of fMetRO, a previously unrecognized marker of tissue damage accumulation following ROS production. Such observations, taken together with the characterization of fRMsr, an efficient fMetRO reductase found in prokaryotes and unicellular eukaryotes (Lin et al., 2007b), led to the development of TYfR, the first version of fMetRO sensor with a clinical potential.

To develop the TYfR, we introduced *fRMsr* and *cpYFP* genes into a vector with mutant *Trx1* in which the resolving cysteine 35 was substituted with serine. We found that TYfR is a ratiometric fluorescent sensor with a high specificity toward fMetRO, but not in response to either fMetSO or strong oxidizing reagents, allowing for the specific quantification of fMetRO by measuring RFI at the two excitation peaks (500 nm/430 nm). TYfR also has unique physiochemical properties, which led us to conduct the experiments in a specific condition wherein a fully reduced sensor was used to measure fMetRO level of various samples at pH 7.5.

Notably, the fMetRO sensitivity of the sensor (250 nM–500 nM) was much higher than that of MetROx, a previously designed pMetRO sensor (Tarrago et al., 2012); such a high sensitivity of the TYfR supports the detection of fMetRO in the physiological range of concentrations. When transfected into 293FT cells, TYfR sensor could also monitor fMetRO *in vivo*. We also observed that TYfR displayed specific and differential RFIs according to its subcellular localization, suggesting that subcellular level of fMetRO may be differentially regulated; ER and mitochondria may be more fMetRO-enriched as compared to cytosol and nucleus. Indeed, this notion is concordant with recent studies in which mitochondria and ER were proposed as organelles with the most prevalent ROS production (Malinouski et al., 2011; Yoboue et al., 2018). However, we do not rule out the possibility that such differences attribute to the unique subcellular environment (e.g. subcellular GSH level and pH), which should be further addressed. This observation, taken together with 1) quantification of ROS in intact cells or cell lysates treated with oxidative stressor, 2) steady state and real-time measurement of fMetRO in two mouse models of systemic ROS production, suggest that TYfR and its substrate can also be employed as a molecular biology platform for dynamic monitoring of physiological and pathological production of (sub)cellular ROS level with a high sensitivity and reproducibility, which would be greatly useful for studying ROS-related signaling pathway and molecular dynamics *in vitro* and *in vivo*.

ROS biology has long been implicated in the pathology of the cardiovascular system (Panth et al., 2016). For example, aberrant local production of ROS results in protein and lipid oxidation, leading to subsequent endothelial dysfunction and inflammation, which further increases ROS levels at the site of pathology (Vichova and Motovska, 2013). Such a positive feedforward event has been shown to increase the incidence of ACS, one of the most severe cardiovascular diseases. Paradoxically, it has also been shown that clinical strategies, including PCI and fibrinolytic therapy for restoration of blood flow in ACS patients, also transiently provoke the positive loop, by causing a drastic spike in ROS production at the reperfusion site, which could lead to severe tissue damage in cardiomyocytes and subsequent necrotic cell death. This pathological phenomenon is referred to as ischemia reperfusion injury, which should be carefully monitored after therapy (Zhou et al., 2018). In this context, characterizing biomarkers of tissue damage

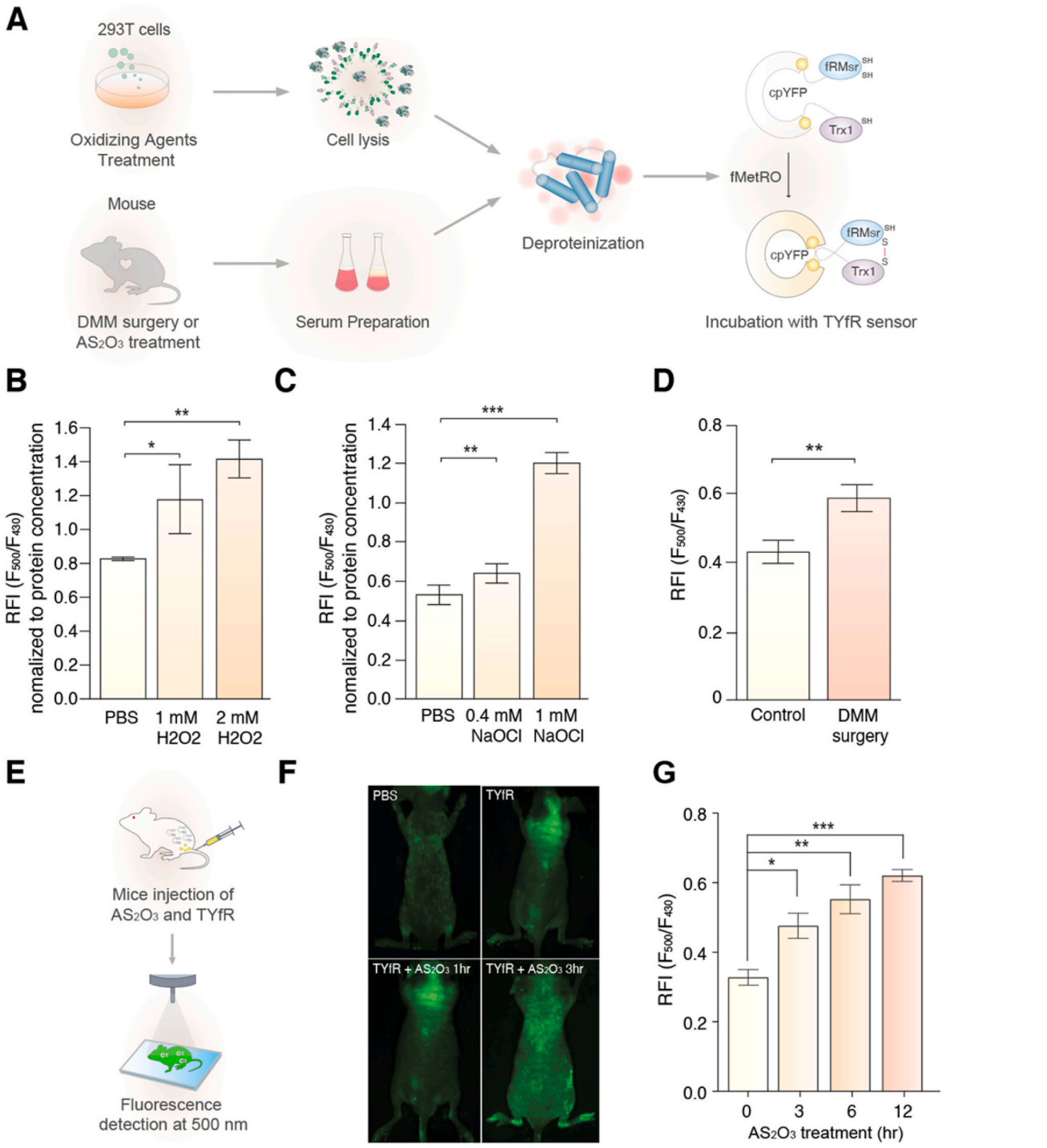

Fig. 4. Application of TYfR for detection of fMetRO in cell lysates and serum from HEK293FT cells and mice challenged with oxidative stress, respectively
(A) Experimental procedure for TYfR application in fMetRO measurement in cell lysates or mouse serum
(B) TYfR response to fMetRO in HEK293FT cell lysates treated with H_2O_2. The lysates were first deproteinized using trichloroacetic acid. The fluorescence intensity ratio (F_{500}/F_{430}) was normalized to total protein concentration. Data are presented as mean ± SD from six replicates
(C) TYfR response to fMetRO in HEK293FT cell lysates treated with NaOCl. The lysates were deproteinized using trichloroacetic acid prior to the reaction. The fluorescence intensity ratio (F_{500}/F_{430}) was normalized to total protein concentration. Data are presented as mean ± SD from six replicates
(D) Response of TYfR to serum fMetRO of mice that underwent sham or DMM surgery. Fluorescence intensities were normalized to the fluorescence values of the most oxidized and reduced forms of TYfR within each experiment. Data are presented as mean ± SD from three biological replicates
(E and F) *In vivo* multispectral imaging of immune-compromised mice subjected to intravenous injection of TYfR and intraperitoneal injection of As_2O_3 for indicated time periods. Excitation and longpass emission filters with wavelengths of 435–480 nm and 490 nm, respectively, were used
(G) Response of TYfR to serum fMetRO in As_2O_3-treated mice. Fluorescence intensities were normalized to the fluorescence values of the most oxidized and reduced forms of TYfR within each experiment. Data are presented as mean ± SD from three biological replicates.Abbreviations: AS_2O_3, arsenic trioxide; DMM, destabilization of the medial meniscus.

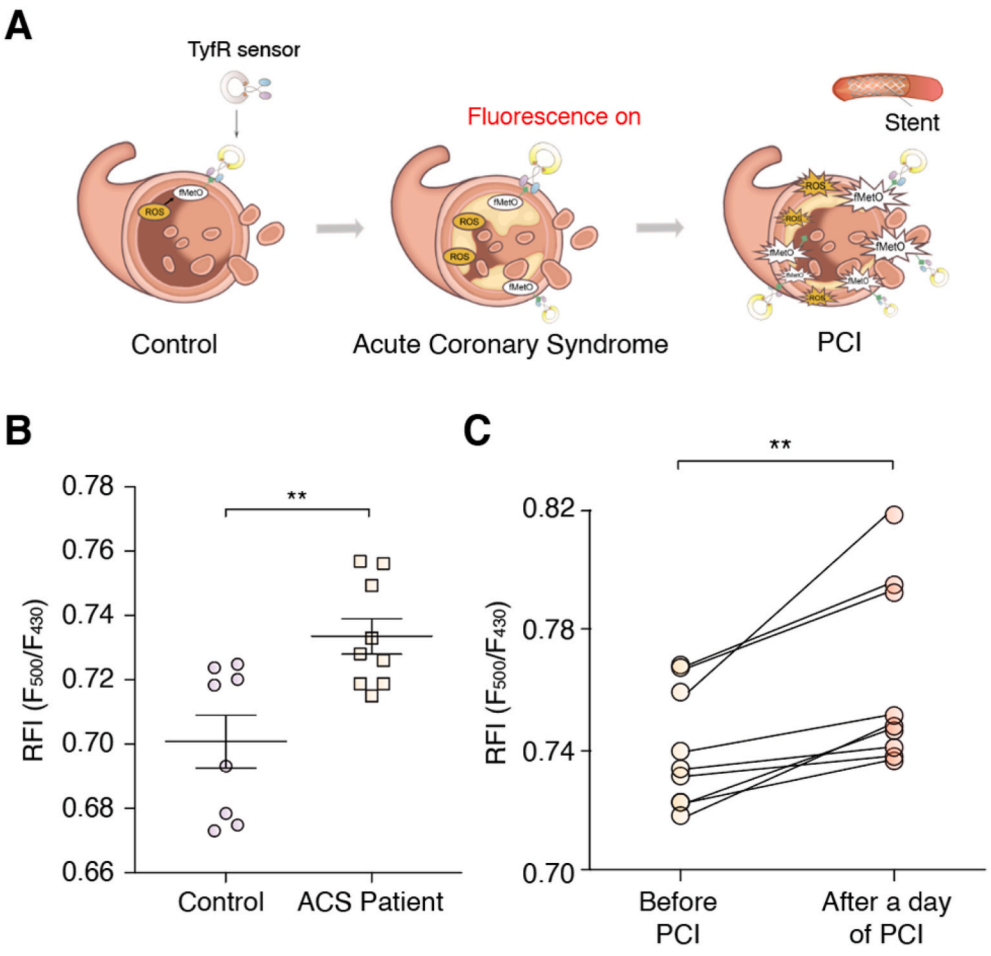

Fig. 5. Clinical usage of TYfR for detecting fMetRO, a biomarker of accumulative tissue damage following oxidative stress from ischemia-reperfusion injury in ACS patients undergoing PCI
(A) Pathogenesis of ACS and ischemia-reperfusion injury in ACS patients undergoing PCI. A significant increase was observed in the serum levels of ROS during ACS development in a positive feedforward manner, leading to serum accumulation of fMetRO (see discussion). Recovery of blood flow following PCI also provoked a drastic rise in the serum level of ROS and fMetRO
(B) Quantification of fMetRO in human serum from 1) eight control individuals having chest pain but with normal coronary arteries and 2) nine patients with ACS undergoing PCI. Each sample was incubated with TYfR (16.5–17.5 μM) and the normalized fluorescence intensity ratio (F_{500}/F_{430}) was obtained, as shown in Fig. 2. The concentration was calculated by interpolation against the standard curve. Each dot represents the mean of three replicates in a sample. Data were analyzed using two-tailed unpaired *t*-test ($p = 0.0042$)
(C) Tracking fMetRO levels in patients before and after PCI. Serum was collected from the subjects described in (a) to (B) one day before and after PCI. All the values were obtained as shown in (a) to (B) and the normalized fluorescence intensity ratio (F_{500}/F_{430}) of the samples of each subject obtained before and after PCI have been connected using lines. Data were analyzed using two-tailed paired *t*-test ($p = 0.0085$).; Abbreviations: ACS, acute coronary syndrome; PCI, percutaneous coronary intervention.

in patients undergoing PCI, followed by the development of a platform with corresponding sensors, are of great importance. Moreover, such a platform can also be employed for testing the efficiency of various antioxidants in ameliorating increased ROS levels in patients undergoing PCI, as well as in patients with other ROS-related diseases.

Our newly developed sensor has the diagnostic potential for measuring the accumulated tissue damage induced by oxidative stress in human pathologies relevant to ROS. This is evident from the observations in which TYfR detects 1) significant enrichment of fMetRO in serum biopsies from ACS patients versus that in control subjects and 2) the dramatic rise in serum levels of fMetRO in ACS patients undergoing PCI.

Finally, we provide the first clinical evidence that MetRO is a promising biomarker for accrued damage induced by oxidative stress in ROS-related human pathologies. This may not only be the consequence of the aberrant ROS production, but also contributes to the pathogenesis of ROS-related diseases, given that intracellular pool of free methionine and its intermediates has been implicated in regulating several metabolic pathways including protein translation, transsulfuration and one carbon pathways (Roy et al., 2020; Shiraki et al., 2014), all of which are tightly associated with cellular responses to aberrant ROS production. In this regard, TYfR sensor may also be a promising tool for expanding our knowledges on how methionine-related pathways and the relevant physiological processes (e.g. glutathione metabolism and epigenetic regulation) can be regulated in the context of ROS-related human diseases.

Therefore, understanding the mechanism of fMetRO regulation and its pathophysiological relevance in the context of various ROS-related diseases will significantly expand the clinical potential of the TYfR sensor.

4. Conclusions

Our TYfR sensor is the first genetically-encoded fluorescent biosensor displaying a remarkable sensitivity and specificity toward fMetRO (free Methionine-R-sulfoxide), allowing for steady state and real-time quantification of ROS and methionine redox status from cells to whole-body level. We believe that the TYfR sensor possesses a greatly relevant potential for studying ROS-related human physiologies and pathologies.

CRediT authorship contribution statement

Dong Wook Choi: conceived the study, investigated to design the experiment, carried out the experiments, supervised all, Writing - original draft, Writing - review & editing. **Yeon Jin Roh:** Conceptualization, the study, investigated to design the experiment, carried out the experiments, Writing - original draft, Writing - review & editing. **Seahyun Kim:** Conceptualization, the study, investigated to design the experiment, carried out the experiments, Writing - original draft, Writing - review & editing. **Hae Min Lee:** investigated to design the experiment. **Minseo Kim:** investigated to design the experiment. **Donghyuk Shin:** investigated to design the experiment, carried out the experiments. **Jong Ho Park:** investigated to design the experiment. **Yongmin Cho:** Writing - review & editing. **Hee Ho Park:** investigated to design the experiment, Writing - review & editing. **Yong Sik Ok:** Writing - review & editing.

Donghyun Kang: provided serum resources for analysis. **Jin-Hong Kim:** provided serum resources for analysis. **Lionel Tarrago:** Conceptualization, the study, Writing - review & editing. **Nika N. Danial:** conceived the study. **Vadim N. Gladyshev:** Conceptualization, the study, Writing - review & editing. **Pil-Ki Min:** conceived the study, provided serum resources for analysis, Writing - original draft, Writing - review & editing. **Byung Cheon Lee:** conceived the study, investigated to design the experiment, supervised all, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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